



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:

C12N 15/86

(11) International Publication Number:

WO 97/42338

(43) International Publication Date: 13 November 1997 (13.11.97)

(21) International Application Number: PCT/US97/07697

(22) International Filing Date: 5 May 1997 (05.05.97)

(30) Priority Data:

08/643,411	6 May 1996 (06.05.96)	US
08/721,327	26 September 1996 (26.09.96)	US

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(81) Designated States: AL, AM, AT, AU, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

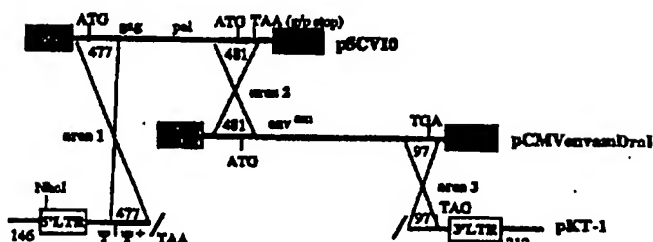
With international search report.

(54) Title: CROSSLESS RETROVIRAL VECTORS

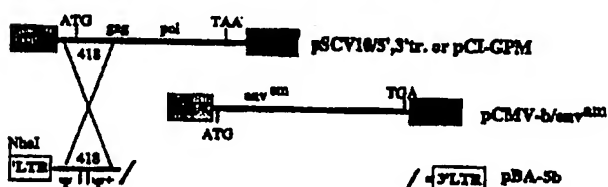
## (57) Abstract

Retroviral vector constructs are described which have a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand synthesis and a 3' LTR, wherein the vector construct lacks retroviral *gag/pol* or *env* coding sequences. In addition, *gag/pol*, and *env* expression cassettes are described wherein the expression cassettes lack a consecutive sequence of more than 8 nucleotides in common. The above-described retroviral vector constructs, *gag/pol* and *env* expression cassettes may be utilized to construct producer cell lines which preclude the formation of replication competent virus.

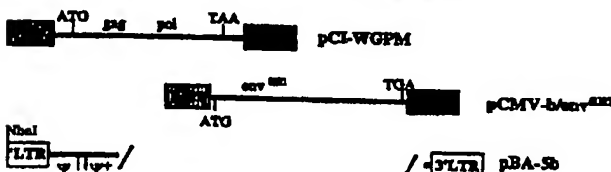
## A. Unmodified retroviral components (three areas of overlap)



## B. Modified retroviral components (overlap 1 reduced, overlap 2 and 3 eliminated)



## C. Modified retroviral components (overlap 1-3 eliminated)



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Description

## CROSSLESS RETROVIRAL VECTORS

5 Technical Field

The present invention relates generally to retroviral vectors for use in gene transfer, and more specifically, to retroviral vectors which are constructed such that the formation of replication competent virus by recombination is precluded.

10 Background of the Invention

Retroviruses are RNA viruses which can replicate and integrate into a host cell's genome through a DNA intermediate. This DNA intermediate, or provirus, may be stably integrated into the host's cellular DNA. Retroviruses are known to be responsible for a wide variety of diseases in both man and animals, including for  
15 example AIDS and a wide variety of cancers.

Although retroviruses can cause disease, they also have a number of properties that lead them to be considered as one of the most promising techniques for genetic therapy of disease. These properties include: (1) efficient entry of genetic material (the vector genome) into cells; (2) an active efficient process of entry into the  
20 target cell nucleus; (3) relatively high levels of gene expression; (4) minimal pathological effects on target cells; and (5) the potential to target particular cellular subtypes through control of the vector-target cell binding and tissue-specific control of gene expression. In using a retrovirus for genetic therapy, a foreign gene of interest may be incorporated into the retrovirus in place of normal retroviral RNA. When the  
25 retrovirus injects its RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself. Expression of this foreign gene within the host results in expression of foreign protein by the host cell.

Most retroviral vector systems which have been developed for gene  
30 therapy are based on murine retroviruses. Briefly, these retroviruses exist in two forms, as proviruses integrated into a host's cellular DNA, or as free virions. The virion form of the virus contains the structural and enzymatic proteins of the retrovirus (including reverse transcriptase), two RNA copies of the viral genome, and portions of the cell's plasma membrane in which is embedded the viral envelope glycoprotein. The genome  
35 is organized into four main regions: the Long Terminal Repeat (LTR), and the *gag*, *pol*, and *env* genes. The LTR may be found at both ends of the proviral genome, is a

composite of the 5' and 3' ends of the RNA genome, and contains *cis*-acting elements necessary for the initiation and termination of transcription. The three genes *gag*, *pol*, and *env* are located between the terminal LTRs. The *gag* and *pol* genes encode, respectively, internal viral structures and enzymatic proteins (such as integrase). The  
5 *env* gene encodes the envelope glycoprotein (designated gp70 and p15e) which confers infectivity and host range specificity of the virus, as well as the "R" peptide of undetermined function.

An important consideration in using retroviruses for gene therapy is the availability of "safe" retroviruses. Packaging cell lines and vector producing cell lines  
10 have been developed to meet this concern. Briefly, this methodology employs the use of two components, a retroviral vector and a packaging cell line (PCL). The retroviral vector contains long terminal repeats (LTRs), the foreign DNA to be transferred and a packaging sequence (*y*). This retroviral vector will not reproduce by itself because the genes which encode structural and envelope proteins are not included within the vector  
15 genome. The PCL contains genes encoding the *gag*, *pol*, and *env* proteins, but does not contain the packaging signal "*y*". Thus, a PCL can only form empty virion particles by itself. Within this general method, the retroviral vector is introduced into the PCL, thereby creating a vector-producing cell line (VCL). This VCL manufactures virion particles containing only the retroviral vector's (foreign) genome, and therefore has  
20 previously been considered to be a safe retrovirus vector for therapeutic use.

There are, however, several shortcomings with the current use of VCLs. One issue involves the generation of "live virus" (*i.e.*, replication competent retrovirus; RCR) by the VCL. Briefly, RCR can be produced in conventional producer cells when:  
25 (1) The vector genome and the helper genomes recombine with each other; (2) The vector genome or helper genome recombines with homologous cryptic endogenous retroviral elements in the producer cell; or (3) Cryptic endogenous retroviral elements reactivate (*e.g.*, xenotropic retroviruses in mouse cells).

Another issue is the propensity of mouse based VCLs to package endogenous retrovirus-like elements (which can contain oncogenic gene sequences) at  
30 efficiencies close to that with which they package the desired retroviral vector. Such elements, because of their retrovirus-like structure, are transmitted to the target cell to be treated at frequencies that parallel its transfer of the desired retroviral vector sequence.

A third issue is the ability to make sufficient retroviral vector particles at  
35 a suitable concentration to: (1) treat a large number of cells (*e.g.*,  $10^8$  -  $10^{10}$ ); and (2) manufacture vector particles at a commercially viable cost.



In order to construct safer PCLs, researchers have generated deletions of the 5' LTR and portions of the 3' LTR of helper elements (*see*, Miller and Buttimore, *Mol. Cell. Biol.* 6:2895-2902, 1986). When such cells are used, two recombination events are necessary to form the wild-type, replication competent genome. Nevertheless, results from several laboratories have indicated that even when several deletions are present, RCR may still be generated (*see*, Bosselman et al., *Mol. Cell. Biol.* 7:1797-1806, 1987; Danos and Mulligan, *Proc. Nat'l. Acad. Sci. USA* 81:6460-6464, 1988). In addition, cell lines containing both 5' and 3' LTR deletions which have been constructed have thus far not proven useful since they produce relatively low titers (Dougherty et al., *J. Virol.* 63:3209-3212, 1989).

One of the more recent approaches to constructing safer packaging cell lines involves the use of complementary portions of helper virus elements, divided among two separate plasmids, one containing *gag* and *pol*, and the other containing *env* (*see*, Markowitz et al., *J. Virol.* 62:1120-1124; and Markowitz et al., *Virology* 167:600-606, 1988). One benefit of this double-plasmid system is that three recombination events are required to generate a replication competent genome. Nevertheless, these double-plasmid vectors have also suffered from the drawback of including portions of the retroviral LTRs, and therefore remain capable of producing infectious virus.

The present invention overcomes the difficulties of recombination and low titer associated with many of the prior packaging cell lines, and further provides other related advantages.

#### Summary of the Invention

Briefly stated, the present invention provides compositions and methods for the construction of packaging cell lines which preclude the formation of RCR by homologous recombination. Within one aspect of the invention, recombinant retroviral vector constructs (RETROVECTOR™) are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR, wherein the retroviral vector construct lacks *gag/pol* and *env* coding sequences. Within one embodiment of the invention, the retroviral vector construct lacks an extended packaging signal. Within one embodiment, the retroviral vector construct lacks a retroviral nucleic acid sequence upstream of the 5' LTR. Within a preferred embodiment, the retroviral vector constructs lack an *env* coding sequence upstream of the 5' LTR. Within another embodiment, the retroviral vector constructs lack an *env* coding and/or untranslated *env* sequence upstream of the 3' LTR.

Retroviral vector constructs of the present invention may be constructed from one or more retroviruses, including, for example, a wide variety of amphotropic, ecotropic, xenotropic, and polytropic viruses (*see e.g.*, Figures 17A, B, and C).

As noted above, retroviral vector constructs of the present invention  
5 include one or more heterologous sequences. Within certain embodiments of the invention, the retroviral vector construct further comprising a heterologous sequence that is at least x kb in length, wherein x is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7 and 8. Within one embodiment, the heterologous sequence is a gene encoding a cytotoxic protein, such as, for example, ricin, abrin, diphtheria toxin, cholera toxin,  
10 gclonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A. Within other embodiments the heterologous sequence may be an antisense sequence, or an immune accessory molecule. Representative examples of immune accessory molecules include IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, and IL-14. Particularly preferred immune accessory molecules may be selected  
15 from the group consisting of IL-2, IL-12, IL-15 and gamma-interferon, or the group consisting of ICAM-1, ICAM-2, b-microglobulin, LFA3, HLA class I and HLA class II molecules.

Within other embodiments of the invention, the heterologous sequence may encode a gene product that activates a compound with little or no cytotoxicity into  
20 a toxic product. Representative examples of such gene products include type I thymidine kinases such as HSVTK and VZVTK, as well as other prodrug-converting enzymes such as cytosine deaminase. Within another embodiment, the heterologous sequence may be a ribozyme. Within yet other embodiments, the heterologous sequence is a replacement gene, which encode proteins such as Factor VIII, ADA,  
25 HPRT, CF and the LDL Receptor. Within other embodiments, the heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

Within other aspects of the present invention, *gag/pol* expression cassettes are provided, comprising a promoter operably linked to a *gag/pol* gene, and a  
30 polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within one embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other aspects *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the expression cassette does not  
35 co-encapsidate with a replication competent virus.

Within another aspect of the present invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a

polyadenylation sequence, wherein a 3' terminal end of the *gag/pol* gene has been deleted without effecting the biological activity of integrase. Within one embodiment, a 5' terminal end of the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within a further embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other embodiments, the 3' terminal end has been deleted so that nucleotides downstream of nucleotide 5751 or any nucleotide between nucleotide 5751 and 5777 of SEQ ID NO: 1 are deleted.

Within other aspects of the present invention, *env* expression cassettes are provided, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 retroviral nucleotides are included upstream of the *env* gene. Within another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein the *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene. Within yet another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted without effecting the biological activity of *env*. Within one embodiment, the 3' terminal end of the gene has been deleted such that a complete R peptide is not produced by the expression cassette. Within a further embodiment, the *env* gene is derived from a type C retrovirus, and the 3' terminal end has been deleted such that the *env* gene includes less than 18 nucleic acids which encode the R peptide. Within a preferred embodiment, the 3' terminal end has been deleted downstream from nucleotide 7748 of SEQ ID NO: 1.

Within various embodiments of the invention, the promoters of the *gag/pol* and *env* expression cassettes described above are heterologous promoters, such as CMV IE, the HVTk promoter, RSV promoter, Adenovirus major-late promoter and the SV40 promoter. Within other embodiments, the polyadenylation sequence is a heterologous polyadenylation sequence, such as the SV40 late poly A Signal and the SV40 early poly A Signal.

Within another aspect of the present invention, packaging cell lines are provided, comprising a *gag/pol* expression cassette and an *env* expression cassette, wherein the *gag/pol* expression cassette lacks a consecutive sequence of greater than 20, preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 consecutive nucleotides which are found in the *env* expression cassette. Within other aspects, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a

consecutive sequence of greater than 20, preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 nucleotides in common. Representative examples of such retroviral vector constructs, *gag/pol* and *env* expression cassettes are described in more detail below.

5           Within yet another aspect of the present invention, producer cell lines are provided comprising a packaging cell line as described above, and a retroviral vector construct. Within another aspect of the present invention, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression  
10 cassette and retroviral vector construct lack a consecutive sequence of greater than eight nucleotides in common.

          Within particularly preferred embodiments of the invention, packaging cell lines are provided which 'mix and match' various elements of the above described retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes.  
15 Briefly, many previous packaging cell lines have three areas of overlap: (1) between the retroviral vector construct and the *gag/pol* expression cassette; (2) between the *gag/pol* expression cassette and the *env* expression cassette; and/or (3) between the *env* expression cassette and the retroviral vector. As described herein, packaging cell lines and producer cell lines with reduced sequence overlap can be produced with no  
20 sequence overlap in area 1, area 2, or, area 3 only, a combination of any two (e.g., no sequence overlap in areas 1 and 2 only, no sequence overlap in areas 1 and 3 only, or no sequence overlap in areas 2 and 3 only), or no sequence overlap in any of the three areas. For example, within one aspect of the present invention producer cell lines are provided comprising a *gag/pol* expression cassette, an *env* expression cassette and a  
25 retroviral vector construct, wherein a 3' terminal end of a *gag/pol* gene encoded within said *gag/pol* expression cassette lacks homology with a 5' terminal end of an *env* gene encoded within said *env* expression cassette, and wherein a 3' terminal end of said *env* gene lacks homology with said retroviral vector construct, with the proviso that said retroviral vector construct overlaps with at least 4 nucleotides (and as many as 8, 10, 15,  
30 20, or more nucleotides) of a 5' terminal end of said *gag/pol* gene encoded within said *gag/pol* expression cassette. As utilized herein, the phrase "lack homology" means that the two cassettes or cassette and construct lack at least 3 or 4, and preferably more than 8, 10, 15 or 20 consecutive nucleotides in common.

          Within other aspects of the invention, methods of producing a packaging  
35 cell line are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette as described above into an animal cell; (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env*

expression cassette into said selected cell, and (d) selecting a cell which expresses high levels of *env* and thereby producing the packaging cell. Within other aspects of the invention, the *env* expression cassette may be introduced into the cell first, followed by the *gag/pol* expression cassette. Within other aspects, methods are provided for  
5 producing recombinant retroviral particles comprising the step of introducing a retroviral vector construct into a packaging cell as described above. Within preferred embodiments, the retroviral vector construct is one of the retroviral vector constructs described above. As noted above, within any of the methods described herein not all areas of sequence overlap must be eliminated. Thus, within certain embodiments  
10 sequence overlap is not eliminated, for example, between the retroviral vector construct and the *gag/pol* expression cassette.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures  
15 or compositions (e.g., plasmids, etc.), and are therefore incorporated by reference in their entirety.

#### Brief Description of the Drawings

- Figure 1 is a schematic illustration of pKS2+*Eco*571-LTR(+).  
20 Figure 2 is a schematic illustration of pKS2+*Eco*571-LTR(-).  
Figure 3 is a schematic illustration of pKS2+LTR-*Eco*RI.  
Figure 4 is a schematic illustration of pR1.  
Figure 5 is a schematic illustration of pR2.  
Figure 6 is a schematic illustration of pKT1.  
25 Figure 7 is a schematic illustration of pRI-HIVenv.  
Figure 8 is a schematic illustration of pR2-HIVenv.  
Figure 9 is a representative "prewobble" sequence for a MoMLV *gag/pol* (see also SEQ I.D. Nos. 11 and 12).  
Figure 10 is a representative "wobble" sequence for a MoMLV *gag/pol*  
30 (see also SEQ. I.D. Nos. 9 and 10).  
Figure 11 is a schematic illustration of pHCMV-PA.  
Figure 12 is a schematic illustration of pCMV *gag/pol*.  
Figure 13 is a schematic illustration of pCMVgpSma.  
Figure 14 is a schematic illustration of pCMVgp-X.  
35 Figure 15 is a schematic illustration of pCMV env-X.  
Figure 16 is a schematic illustration of pRgpNeo.

Figures 17A, B and C comprise a table which sets forth a variety of retroviruses which may be utilized to construct the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present invention.

Figure 18 is a schematic illustration of pCMV Envam-Eag-X-less.

5 Figure 19A is a diagrammatic illustration of a "wobble" -*gag* construct. Figure 19B is a diagrammatic illustration of a "normal" -*gag* construct.

Figure 20 is a description of all modifications carried out on retroviral vector as shown in A), resulting in the cross-less retroviral vector shown in B). The cross-less retroviral backbone cloned into a prokaryotic vector is called pBA-5.

10 Figure 21 depicts retroviral amphotropic envelope constructs starting with the pCMVenvAMDral at the top of the page and modifications thereof. The exact modifications in the envelope constructs are described in the examples.

Figures 22A, 22B and 22C are schematics showing retrovirus with three regions (A), one region (B) and no region (C) of sequence overlap.

15

#### Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

20 "Retroviral vector construct" refers to an assembly which is, within preferred embodiments of the invention, capable of directing the expression of a sequence(s) or gene(s) of interest. Briefly, the retroviral vector construct must include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein  
25 (e.g., cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement gene), or which are useful as a molecule itself (e.g., as a ribozyme or antisense sequence). Alternatively, the heterologous sequence may merely be a "stuffer" or "filler" sequence, which is of a size sufficient to allow production of viral particles containing the RNA genome. Preferably, the heterologous sequence is at least  
30 1, 2, 3, 4, 5, 6, 7 or 8 kB in length.

The retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally,  
35 the retroviral vector construct may also include selectable markers such as Nco, TK, hygromycin, phleomycin, histidinol, human placental Alkaline Phosphatase, NGFR or

DHFR, as well as one or more specific restriction sites and a translation termination sequence.

"Expression cassette" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence. Within preferred embodiments of the invention, both the promoter and the polyadenylation sequence are from a source which is heterologous to the helper elements (*i.e.*, *gag/pol* and *env*). Expression cassettes of the present invention may be utilized to express a *gag/pol* gene or an *env* gene. In addition, the expression cassettes may also be utilized to express one or more heterologous sequences either from a *gag/pol* and/or *env* expression cassette, or from a entirely different expression cassette.

Within preferred embodiments of the invention, the expression cassettes described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication).

#### Preparation of Retroviral vector constructs, *Gag/Pol* Expression Cassettes and *Env* Expression Cassettes

As noted above, the present invention provides compositions and methods for constructing packaging cells which preclude the formation of replication competent virus by homologous recombination. The following sections describe the preparation of retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes.

##### 1. Construction of retroviral vector constructs

Within one aspect of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTR's may be readily identified in the provirus due to their precise duplication at either end of the genome.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, tRNA binds to a retroviral tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to 5' and 3' LTRs, a tRNA binding site, and an origin of second strand DNA synthesis, retroviral vector constructs of the present invention also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Retroviral vector constructs of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include those set forth below in Figures 17A, B and C (*see* RNA Tumor Viruses at pages 2-7), as well as a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2 and NZB<sub>9-1</sub> (*see* O'Neill et al., *J. Vir.* 53:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see* Kelly et al., *J. Vir.* 45(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Particularly preferred retroviruses for the preparation or construction of retroviral vector constructs of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus, Gibbon Ape Leukemia Virus, Mason Pfizer Monkey Virus, and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol.* 19:19-25, 1976), Abelson (ATCC No.



VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659; and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral vector constructs, packaging cells, or producer cells of the present invention given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 82:488, 1985). Further, within certain embodiments of the invention, portions of the retroviral vector construct may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus. Similarly, portions of a packaging cell line may be derived from different viruses (*e.g.*, a *gag/pol* expression cassette may be constructed from a Moloney Murine Leukemia Virus, and an *env* expression cassette from a Mason Pfizer Monkey virus).

As noted above, within various aspects of the present invention, retroviral vector constructs are provided which have packaging signals, and which lack both *gag/pol* and *env* coding sequences. As utilized within the context of the present invention, a packaging signal should be understood to refer to that sequence of nucleotides which is not required for synthesis, processing or translation of viral RNA or assembly of virions, but which is required in *cis* for encapsidation of genomic RNA (*see* Mann et al., *Cell* 33:153-159, 1983; RNA Tumor Viruses, Second Edition, *supra*). Further, as utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to refer to retrovectors which contain less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retroviral vector construct. Representative examples of such retroviral vector constructs are set forth in more detail below and in Example 1.

As an illustration, within one embodiment of the invention construction of retroviral vector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing retroviral vector constructs which lack an extended

packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence (counting from the 5' LTR cap site) from approximately nucleotide 144 of SEQ. I.D. No. 1, up through the *Pst* I site (nucleotide 567 of SEQ. I.D. No. 1). The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1040. Thus, within this embodiment retroviral vector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal downstream of nucleotide 567.

Within other embodiments of the invention, retroviral vector constructs are provided wherein the packaging signal that extends into, or overlaps with, retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated downstream of the start of the *gag/pol* gene (nucleotide 621 of SEQ ID NO: 1). Within preferred embodiments of the invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615 or 617 of SEQ ID NO: 1.

Within other aspects of the invention, retroviral vector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene (e.g., for MoMLV, beyond nucleotide 621 of SEQ ID NO: 1). When such retroviral vector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*. Such *gag/pol* expression cassettes are described in more detail below in section 2, and in Example 3.

Within certain embodiments, the packaging signal that extends beyond the start of the *gag/pol* gene was modified in order to contain one, two or more stop codons within the *gag/pol* reading frame. Most preferably, one of the stop codons eliminates the start site and/or has two or three base pair substitutions. One representative example of such modifications is provided below in Example 9.

Within other aspects of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence upstream of the 5' LTR" should be understood to mean that the

retrovector plasmid construct contains less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in a retrovirus which is homologous to the retroviral vector construct, upstream of and/or contiguous with the 5' LTR. Within preferred embodiments, the retrovector plasmid constructs do not contain an *env* coding sequence (as discussed below) upstream of the 5' LTR. A particularly preferred embodiment of such retrovector plasmid constructs is set forth in more detail below in Example 1.

Within a further aspect of the present invention, retrovector plasmid constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome. A representative example of such a retroviral vector construct is set forth in more detail below in Example 1.

Within other aspects of the present invention, retrovector plasmid constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain envelope sequences upstream of the 3' LTR. As utilized within this context, the term "envelope sequence" should be understood to mean envelope coding as well as flanking untranslated sequences. A representative example of such a retroviral vector construct is set forth in more detail below in Example 9.

## 2. Construction of gag/pol expression cassettes

As noted above, the present invention also provides a variety of *gag/pol* expression cassettes which, in combination with the retroviral vector constructs and *env* expression cassettes of the present invention, enable the construction of packaging cell lines and producer cell lines which preclude the formation of replication competent virus. Briefly, retroviral *gag/pol* genes contain a *gag* region which encodes a variety of structural proteins that make up the core matrix and nucleocapsid, and a *pol* region which contains genes which encode (1) a protease for the processing of *gag/pol* and *env* proteins, (2) a reverse transcriptase polymerase, (3) an RNase H, and (4) an integrase, which is necessary for integration of the retroviral provector into the host genome. Although retroviral *gag/pol* genes may be utilized to construct the *gag/pol* expression cassettes of the present invention, a variety of other non-retroviral (and non-viral) genes may also be utilized to construct the *gag/pol* expression cassette. For example, a gene

which encodes retroviral RNase H may be replaced with genes which encode bacterial (e.g., *E. coli* or *Thermus thermophilus*) RNase H. Similarly, a retroviral integrase gene may be replaced by other genes with similar function (e.g., yeast retrotransposon TY3 integrase).

5           Within one aspect of the invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Briefly, as noted above, in wild-type retrovirus the extended packaging signal of the retrovirus overlaps with sequences which encode  
10 gag and pol. Thus, in order to eliminate the potential of crossover between the retroviral vector construct and the *gag/pol* expression cassette, as well as to eliminate the possibility of co-encapsidation of the *gag/pol* expression cassette and replication competent virus or retroviral vector constructs, sequences of overlap should be eliminated. Within one embodiment of the invention, elimination of such overlap is  
15 accomplished by modifying the *gag/pol* gene (and more specifically, regions which overlap with the retroviral vector construct, such as the extended packaging signal) to contain codons that are degenerate (i.e., that "wobble") for gag. In particular, within preferred embodiments of the invention codons are selected which encode biologically active gag/pol protein (i.e., capable of producing a competent retroviral particle, in  
20 combination with an *env* expressing element, and a RNA genome), and which lack any packaging signal sequence, including in particular, extended packaging signal sequence. As utilized herein, the phrase "lacks any retroviral packaging signal sequence" should be understood to mean that the *gag/pol* expression cassette contains less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8  
25 consecutive nucleotides which are identical to a sequence found in a retroviral packaging signal (e.g., in the case of MoMLV, extending up and through the *Xho* I site at approximately nucleotide number 1561). A particularly preferred example of such modified codons which are degenerate for gag is shown in Figure 10, and in Example 3, although the present invention should not be so limited. In particular, within other  
30 embodiments, at least 25, 50, 75, 100, 125 or 135 gag codons are modified or "wobbled" from the native gag sequence within the *gag/pol* expression cassettes of the present invention.

          In addition to eliminating overlap between the retroviral vector construct and the *gag/pol* gene, it is also preferable to eliminate any potential overlap between the  
35 *gag/pol* gene and the *env* gene in order to prohibit the possibility of homologous recombination. This may be accomplished in at least two principal ways: (1) by deleting a portion of the *gag/pol* gene which encodes the integrase protein, and in

particular, that portion of the gene which encodes the integrase protein which overlaps with the *env* coding sequence, or (2) by selecting codons which are degenerate for integrase and/or *env*.

Thus, within one aspect of the present invention *gag/pol* expression  
5 cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence or signal, wherein a 3' terminal end of the gene has been deleted without effecting the biological activity of the integrase. (The biological activity of integrase may be readily determined by detection of an integration event, either by DNA analysis or by expression of a transduced gene; *see* Roth et al., *J. Vir.*  
10 65(4):2141-2145, 1991.) As an example, in the Murine Leukemia Virus MoMLV (SEQ ID. NO. 1), the *gag/pol* gene is encoded by nucleotides 621 through 5834. Within this sequence, the protein integrase is encoded by nucleotides 4610 through nucleotide 5834. A portion of the *gag/pol* sequence which encodes integrase also encodes *env* (which begins at nucleotide 5776). Thus, within one embodiment of the invention, the  
15 3' terminal end of the *gag/pol* gene is deleted or truncated in order to prevent crossover with the *env* gene. without effecting the biological activity of the integrase. Within other preferred embodiments, the *gag/pol* gene is deleted at any nucleotide downstream (3') from the beginning of the integrase coding sequence, and preferably prior to the start of the *env* gene sequence. Within one embodiment, the sequence encoding *gag/pol*  
20 is a MoMLV sequence, and the *gag/pol* gene is deleted at any nucleotide between nucleotides 4610 and 5776 (of SEQ. I.D. No. 1), including for example, at nucleotides 5775, 5770, 5765, 5760, 5755, 5750.

Within other embodiments of the invention, the *gag/pol* expression cassette contains sequences encoding *gag/pol* (and including integrase), while lacking  
25 any sequence found in an *env* gene. The phrase "lacking any sequence found in an *env* gene" should be understood to mean that the *gag/pol* expression cassette does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably less than 8 consecutive nucleotides which are identical to an *env* sequence, and preferably which are found in an *env* expression cassette which will be utilized  
30 along with the *gag/pol* expression cassette to form a packaging cell. Such expression cassettes may be readily prepared by selecting codons which are degenerate for integrase, and which do not encode biologically active *env*. (*See Morgenstern and Land, Nuc. Acids Res. 18:3587-3596, 1990.*)

Within other embodiments of the invention, the *gag/pol* expression  
35 cassette contains a heterologous promoter, and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters or polyadenylation sequences refers to promoters or polyadenylation sequences which are from a different source

from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the Cytomegalovirus Immediate Early ("CMV IE") promoter, the Herpes Simplex Virus Thymidine Kinase ("HSVTK") promoter, the Rous Sarcoma Virus ("RSV") promoter, the Adenovirus major-late promoter and the SV 40 promoter. Representative examples of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal.

Within preferred aspects of the present invention, *gag/pol* expression cassettes such as those described above will not co-encapsidate along with a replication competent virus. One representative method for determination of co-encapsidation is set forth below in Example 8.

### 3. Construction of *env* expression cassettes

Within other aspects of the present invention, *env* expression cassettes are provided which, in combination with the *gag/pol* expression cassettes and retroviral vector constructs described above, preclude formation of replication competent virus by homologous recombination, as well as to confer a particular specificity of the resultant vector particle (*e.g.*, amphotropic, ecotropic, xenotropic or polytropic; *see* Figure 17, as well as the discussion above). Briefly, in a wild-type retrovirus the *env* gene encodes two principal proteins, the surface glycoprotein "SU" and the transmembrane protein "TM", which are translated as a polyprotein, and subsequently separated by proteolytic cleavage. Representative examples of the SU and TM proteins are the gp120 protein and gp41 protein in HIV, and the gp70 protein and p15c protein in MoMLV. In some retroviruses, a third protein designated the "R" peptide of undetermined function, is also expressed from the *env* gene and separated from the polyprotein by proteolytic cleavage. In the Murine Leukemia Virus MoMLV, the R peptide is designated "p2".

A wide variety of *env* expression cassettes may be constructed given the disclosure provided herein, and utilized within the present invention to preclude homologous recombination. Within one aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein no more than 6, 8, 10, 15, or 20 consecutive retroviral nucleotides are included upstream (5') of and/or contiguous with said *env* gene. Within other aspects of the invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein the *env* expression cassette does not contain a consecutive sequence of greater than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 or 6 consecutive nucleotides which are found in a *gag/pol*

gene, and in particular, in a *gag/pol* expression cassette that will be utilized along with the *env* expression cassette to create a packaging cell line.

Within another aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted without effecting the biological activity of *env*. As utilized herein, the phrase "biological activity of *env*" refers to the ability of envelope protein to be expressed on the surface of a virus or vector particle, and to allow for a successful infection of a host cell. One practical method for assessing biological activity is to transiently transfect the *env* expression cassette into a cell containing a previously determined functional *gag/pol* expression cassette, and a retroviral vector construct which expresses a selectable marker. Another method for assessing biological activity is to either stably transfect the *env* expression cassette together with a retroviral vector construct coding for a selectable marker into a cell containing a previously determined functional *gag/pol* expression cassette, or, transducing this *gag/pol* expressing cell in a transient and/or stable manner with a retroviral vector coding for the *env* gene and a selectable marker. A biologically functional *env* expression cassette will allow vector particles produced in that transfected cell, to transmit the selectable marker to a naive sensitive cell such that it becomes resistant to the marker drug selection. Within a preferred embodiment of the invention, the 3' terminal end of the *env* gene is deleted or truncated such that a complete R peptide is not produced by the expression cassette. In the representative example of MoMLV, sequence encoding the R peptide (which begins at nucleotide 7734) is deleted, truncated, or, for example, terminated by insertion of a stop codon at nucleotide 7740, 7745, 7747, 7750, 7755, 7760, 7765, 7770, 7775, 7780, or any nucleotide in between.

Within another aspect of the present invention, *env* expression cassettes are provided which contain a heterologous promoter, a heterologous leader sequence and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters, leaders or polyadenylation sequences refers to sequences which are from a different source from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the CMV IE promoter, the HSVTK promoter, the RSV promoter, the Adenovirus major-late promoter and the SV 40 promoters. Representative examples of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal, and the bovine growth hormone termination/polyadenylation sequence. Preferably any such termination/

polyadenylation sequence will not have any 10 bp stretch which has more than 80% homology to a retroviral construct.

Envelope expression cassettes that contain no MoMLV noncoding sequences can also be created. For example, analogous to the 3' end modifications described in example 12, noncoding bases on the 5' of envelope prior to the start AUG codon can be deleted as described in Example 4. Another method of 5' end modification is to substitute the 5' untranslated RNA leader of MoMLV envelope with an alternate leader. The 5' untranslated RNA sequence can be a leader from another protein or an entirely synthetic leader. The leader may also contain one or more introns. The only requirements for the leader are that it contains a Kozak sequence sufficient for efficient translation of the amphotropic envelope. Representative leader sequences may also include untranslated RNA leaders for envelope proteins from other viruses. Examples of these include Vesicular Stomatitis Virus -G protein (VSV-g), Herpes Simplex Virus(HSV) gB protein, or HSV-gD protein. The 5' untranslated leader sequence is inserted so that it spans the sequence between the eucaryotic promoter start site and the amphotropic envelope start codon.

#### HETEROLOGOUS SEQUENCES

As noted above, the retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes of the present invention may contain (and express) one or more heterologous sequences. As utilized within the context of the present invention, it should be understood that the heterologous sequence need not code for a particular protein, but may be merely included in order to improve efficiency of viral particle production. In this regard, heterologous sequences of at least 1 kb or greater are particularly preferred.

A wide variety of heterologous sequences may be utilized within the context of the present invention, including for example, cytotoxic genes, antisense sequences, sequences which encode gene products that activate a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product, sequences which encode immunogenic portions of disease-associated antigens and sequences which encode immune accessory molecules. Representative examples of cytotoxic genes include the genes which encode proteins such as ricin (Lamb et al., *Eur. J. Biochem.* 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem.* 198:723-732, 1991; Evensen, et al., *J. of Biol. Chem.* 266:6848-6852, 1991; Collins et al., *J. of Biol. Chem.* 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc.* 309:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem.* 260:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature* 306:551-557, 1983; Sanchez & Holmgren, *PNAS* 86:481-485, 1989), gelonin



(Stirpe et al., *J. Biol. Chem.* 255:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther.* 21:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J.* 203:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys.* 200:418-425, 1980; Irvin, *Arch. Biochem. & Biophys.* 169:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS* 84:4364-4368, 1987; Jackson et al., *Microb. Path.* 2:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem.* 262:8707-8711, 1987).

Within further embodiments of the invention, antisense RNA may be utilized as a cytotoxic gene in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences may be utilized to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA.

15 Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

Within other embodiments of the invention, antisense sequences are provided which inhibit, for example, tumor cell growth, viral replication, or a genetic disease by preventing the cellular synthesis of critical proteins needed for cell growth. Examples of such antisense sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys.* 253:214-220, 1987; Bzik et al., *PNAS* 84:8360-8364, 1987), antisense HER2 (Coussens et al., *Science* 230:1132-1139, 1985), antisense ABL (Fainstein, et al., *Oncogene* 4:1477-1481, 1989), antisense Myc (Stanton et al., *Nature* 310:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

Within other aspects of the invention, retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes are provided which direct the expression of a gene product that activates a compound with little or no cytotoxicity (i.e., a "prodrug") into a toxic product. Representative examples of such gene products include varicella zoster virus thymidine kinase (VZVTK), herpes simplex virus thymidine kinase (HSVTK) (Field et al., *J. Gen. Virol.* 49:115-124, 1980; Munir et al., *Protein Engineering* 7(1):83-89, 1994; Black and Loeb, *Biochem* 32(43):11618-11626, 1993), and *E. coli* guanine phosphoribosyl transferase (see U.S. Patent Application Serial No. 08/155,944, entitled "Compositions and Methods for Utilizing Conditionally Lethal Genes," filed November 18, 1993; see also WO 93/10218 entitled "Vectors Including Foreign Genes and Negative Selection Markers", WO 93/01281 entitled "Cytosine Deaminase Negative Selection System for Gene Transfer Techniques and

Therapies", WO 93/08843 entitled "Trapped Cells and Use Thereof as a Drug", WO 93/08844 entitled "Transformant Cells for the Prophylaxis or Treatment of Diseases Caused by Viruses, Particularly Pathogenic Retroviruses", and WO 90/07936 entitled "Recombinant Therapies for Infection and Hyperproliferative Disorders.") Within  
5 preferred embodiments of the invention, the retroviral vector constructs direct the expression of a gene product that activates a compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby affecting localized therapy to the pathogenic agent (*see* WO 94/13304).

Within one embodiment of the invention, retroviral vector constructs are  
10 provided which direct the expression of a HSVTK gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the vector construct causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed  
15 to a drug such as ganciclovir, acyclovir or its analogues (FIAU, FIAC, DHPG). Such drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir and FIAU triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (*see* Borrelli et al., *Proc.*  
20 *Natl. Acad. Sci. USA* 85:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

Within further aspects of the present invention, retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present  
25 invention may also direct the expression of one or more sequences which encode immunogenic portions of disease-associated antigens. As utilized within the context of the present invention, antigens are deemed to be "disease-associated" if they are either associated with rendering a cell (or organism) diseased, or are associated with the disease-state in general but are not required or essential for rendering the cell diseased.  
30 In addition, antigens are considered to be "immunogenic" if they are capable, under appropriate conditions, of causing an immune response (either cell-mediated or humoral). Immunogenic "portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen.

A wide variety of "disease-associated" antigens are contemplated within  
35 the scope of the present invention, including for example immunogenic, non-tumorigenic forms of altered cellular components which are normally associated with tumor cells (*see* WO 93/10814). Representative examples of altered cellular

components which are normally associated with tumor cells include ras\* (wherein "\*" is understood to refer to antigens which have been altered to be non-tumorigenic), p53\*, Rb\*, altered protein encoded by Wilms' tumor gene, ubiquitin\*, mucin, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, Platelet Derived Growth Factor ("PDGF") receptor, insulin receptor, Epidermal Growth Factor ("EGF") receptor, and the Colony Stimulating Factor ("CSF") receptor.

"Disease-associated" antigens should also be understood to include all or portions of various eukaryotic, prokaryotic or viral pathogens. Representative examples of viral pathogens include the Hepatitis B Virus ("HBV") and Hepatitis C Virus ("HCV"; see WO 93/15207), Human Papilloma Virus ("HPV"; see WO 92/05248; WO 90/10459; EPO 133,123), Epstein-Barr Virus ("EBV"; see EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus ("FeLV"; see WO 93/09070; EPO 377,842; WO 90/08832; WO 93/09238), Feline Immunodeficiency Virus ("FIV"; U.S. Patent No. 5,037,753; WO 92/15684; WO 90/13573; and JP 4,126,085), HTLV I and II, and Human Immunodeficiency Virus ("HIV"; see WO 93/02805).

Within other aspects of the present invention, the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes described above may also direct the expression of one or more immune accessory molecules. As utilized herein, the phrase "immune accessory molecules" refers to molecules which can either increase or decrease the recognition, presentation or activation of an immune response (either cell-mediated or humoral). Representative examples of immune accessory molecules include a interferon, b interferon, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf et al., *J. Immun.* 46:3074, 1991; Gubler et al., *PNAS* 88:4143, 1991; WO 90/05147; EPO 433,827), IL-13 (WO 94/04680), IL-14, IL-15, GM-CSF, M-CSF-1, G-CSF, CD3 (Krissanen et al., *Immunogenetics* 26:258-266, 1987), CD8, ICAM-1 (Simmons et al., *Nature* 331:624-627, 1988), ICAM-2 (Singer, *Science* 255: 1671, 1992), b-microglobulin (Parnes et al., *PNAS* 78:2253-2257, 1981), LFA-1 (Altmann et al., *Nature* 338: 521, 1989), LFA3 (Wallner et al., *J. Exp. Med.* 166(4):923-932, 1987), HLA Class I, HLA Class II molecules, B7 (Freeman et al., *J. Immun.* 143:2714, 1989), and B7-2. Within a preferred embodiment, the heterologous gene encodes gamma-interferon.

Within preferred aspects of the present invention, the retroviral vector constructs described herein may direct the expression of more than one heterologous sequence. Such multiple sequences may be controlled either by a single promoter, or

preferably, by additional secondary promoters (e.g., Internal Ribosome Binding Sites or "IRBS" also known as Internal Ribosome Entry Sites or "IRES"). Within preferred embodiments of the invention, retroviral vector constructs direct the expression of heterologous sequences which act synergistically. For example, within one  
5 embodiment retroviral vector constructs are provided which direct the expression of a molecule such as IL-15, IL-12, IL-2, gamma interferon, or other molecule which acts to increase cell-mediated presentation in the T<sub>H</sub>1 pathway, along with an immunogenic portion of a disease-associated antigen. In such embodiments, immune presentation and processing of the disease-associated antigen will be increased due to the presence of the  
10 immune accessory molecule.

Within other aspects of the invention, retroviral vector constructs are provided which direct the expression of one or more heterologous sequences which encode "replacement" genes. As utilized herein, it should be understood that the term "replacement genes" refers to a nucleic acid molecule which encodes a therapeutic  
15 protein that is capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect. Representative examples of such genetic defects include disorders in metabolism, immune regulation, hormonal regulation, and enzymatic or membrane associated structural function. Representative examples of diseases caused by such defects include Cystic Fibrosis ("CF"; see Dorin et al., *Nature* 326:614, ),  
20 Parkinson's Disease, Adenosine Deaminase deficiency ("ADA"; Hahma et al., *J. Bact.* 173:3663-3672, 1991), b-globin disorders, Hemophilia A & B (Factor VIII-deficiencies; see Wood et al., *Nature* 312:330, 1984), Gaucher disease, diabetes, forms of gouty arthritis and Lesch-Nylan disease (due to "HPRT" deficiencies; see Jolly et al., *PNAS* 80:477-481, 1983) and Familial Hypercholesterolemia (LDL Receptor mutations; see  
25 Yamamoto et al., *Cell* 39:27-38, 1984).

Sequences which encode the above-described heterologous genes may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode immune accessory molecules may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from  
30 commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative sources sequences which encode the above-noted immune accessory molecules include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF),  
35 ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC

No 67024 (which contains a sequence which encodes Interleukin-1), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6). It will be evident to one of skill in the art that one may utilize either the entire sequence of the protein, or an appropriate portion thereof which encodes the biologically active portion of the protein.

10 Alternatively, known cDNA sequences which encode cytotoxic genes or other heterologous genes may be obtained from cells which express or contain such sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. *See also* PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989 all of which are incorporated by reference herein in their entirety) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

20 Sequences which encode the above-described genes may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California)).

#### PREPARATION OF RETROVIRAL PACKAGING CELL LINES, AND GENERATION OF RECOMBINANT VIRAL PARTICLES

As noted above, the *gag/pol* expression cassettes and *env* expression cassettes of the present invention may be used to generate transduction competent retroviral vector particles by introducing them into an appropriate parent cell line in order to create a packaging cell line, followed by introduction of a retroviral vector construct, in order to create a producer cell line (*see* WO 92/05266). Such packaging cell lines, upon introduction of an N2-type vector construct (Armentano et al., *J. of Vir.* 61(5):1647-1650, 1987) produce a titer of greater than  $10^5$  cfu/ml. and preferably greater than 10-fold, 20-fold, 50-fold, or 100-fold higher titer than similar transduced PA317 cells (Miller and Buttimore, *Mol. and Cell. Biol.* 6(8):2895-2902, 1986).

Within one aspect of the present invention, methods for creating packaging cell lines are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette according into an animal cell, (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env* expression cassette into the selected cell, and (d) selecting a cell which expresses high levels of *env*, and thereby creating the packaging cell. Within other aspects of the present invention, methods for creating packaging cell lines are provided comprising the steps of (a) introducing an *env* expression cassette into an animal cell (b) selecting a cell which expresses high levels of *env*, (c) introducing a *gag/pol* expression cassette into the selected cell, and (d) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, and thereby creating the packaging cell. As utilized herein, it should be understood that "high" levels of *gag/pol* or *env* refers to packaging cells which produce at least *z* times greater *gag/pol* or *env* protein than PA317 cells, wherein *z* is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

A wide variety of animal cells may be utilized to prepare the packaging cells of the present invention, including for example cells obtained from vertebrates, warm-blooded animals, or, mammals such as human, feline, goat, bovine, sheep, caprine, macaque, dog, rat and mouse cells. Particularly preferred cell lines for use in the preparation of packaging cell lines of the present invention are those that lack genomic sequences which are homologous to the retroviral vector construct, *gag/pol* expression cassette and *env* expression cassette to be utilized. Methods for determining homology may be readily accomplished by, for example, hybridization analysis (see *Martin et al.*, *PNAS* 78:4892-4896, 1981; see also WO 92/05266).

Expression cassettes of the present invention may be introduced into cells by numerous techniques, including for example, transfection by various physical methods, such as electroporation, DEAE dextran, lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991), liposomes of several types (see e.g., Wang et al., *PNAS* 84:7851-7855, 1987);  $\text{CaPO}_4$  (Dubensky et al., *PNAS* 81:7529-7533, 1984), DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989), administration of nucleic acids alone (WO 90/11092), or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3: 147-154, 1992).

Producer cell lines (also called vector-producing lines or "VCLs") may then be readily prepared by introducing a retroviral vector construct into a packaging cell line via transfection as described above, or, via transduction. Within preferred

embodiments of the invention, producer cell lines are provided comprising a *gag/pol* expression cassette, an *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 20, preferably 15, more preferably 10, and most preferably 10 or 8 nucleotides in common.

#### PHARMACEUTICAL COMPOSITIONS

Within another aspect of the invention, pharmaceutical compositions are provided, comprising a recombinant viral particle as described above, in combination with a pharmaceutically acceptable carrier or diluent. Such pharmaceutical compositions may be prepared either as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or oral, nasal, vaginal, sub-lingual, inhalant or rectal administration.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a retroviral vector construct or recombinant viral particle in 1 mg/ml HSA, 18.75 mM Tris, pH 7.2, 37.5 mM NaCl and 40.0 mg/ml lactose. In this case, since the recombinant vector represents approximately 1 mg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months.

Pharmaceutical compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a recombinant retroviral vector. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas or epidermal growth factor for breast or other epithelial carcinomas.

Particularly preferred methods and compositions for preserving recombinant viruses are described in U.S. applications entitled "Methods for Preserving Recombinant Viruses" (*see* WO 94/11414).

## METHODS OF ADMINISTRATION

Within other aspects of the present invention, methods are provided for inhibiting or destroying pathogenic agents in a warm-blooded animal, comprising administering to a warm-blooded animal a recombinant viral particle as described  
5 above, such that the pathogenic agent is inhibited or destroyed. Within various embodiments of the invention, recombinant viral particles may be administered *in vivo*, or *ex vivo*. Representative routes for *in vivo* administration include intradermally ("i.d."), intracranially ("i.c."), intraperitoneally ("i.p."), intrathecally ("i.t."), intravenously ("i.v."), subcutaneously ("s.c."), intramuscularly ("i.m.") or even directly  
10 into a tumor.

Alternatively, the cytotoxic genes, antisense sequences, gene products, retroviral vector constructs or viral particles of the present invention may also be administered to a warm-blooded animal by a variety of other methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner  
15 et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991); liposomes of several types (*see e.g.*, Wang et al., *PNAS* 84:7851-7855, 1987); CaPO<sub>4</sub> (Dubensky et al., *PNAS* 81:7529-7533, 1984); DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989); administration of nucleic acids alone  
20 (WO 90/11092); or administration of DNA linked to killed adenovirus (Curicl et al., *Hum. Gene Ther.* 3: 147-154, 1992).

Within a preferred aspect of the present invention, retroviral particles (or retroviral vector constructs alone) may be utilized in order to directly treat pathogenic agents such as a tumor. Within preferred embodiments, the retroviral particles or  
25 retroviral vector constructs described above may be directly administered to a tumor, for example, by direct injection into several different locations within the body of tumor. Alternatively, arteries which serve a tumor may be identified, and the vector injected into such an artery, in order to deliver the vector directly into the tumor. Within another embodiment, a tumor which has a necrotic center may be aspirated, and the  
30 vector injected directly into the now empty center of the tumor. Within yet another embodiment, the retroviral vector construct may be directly administered to the surface of the tumor, for example, by application of a topical pharmaceutical composition containing the retroviral vector construct, or preferably, a recombinant retroviral particle.

35 Within another aspect of the present invention, methods are provided for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising the



steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) infecting the removed cells with a retroviral vector construct which directs the expression of at least one anti-tumor agent, and (c) delivering the infected cells to a warm-blooded animal, such that the growth of the selected tumor is inhibited by immune responses generated against the gene-modified tumor cell. Within the context of the present invention, "inhibiting the growth of a selected tumor" refers to either (1) the direct inhibition of tumor cell division, or (2) immune cell mediated tumor cell lysis, or both, which leads to a suppression in the net expansion of tumor cells. Inhibition of tumor growth by either of these two mechanisms may be readily determined by one of ordinary skill in the art based upon a number of well known methods (*see* U.S. Serial No. 08/032,846). Examples of compounds or molecules which act as anti-tumor agents include immune accessory molecules, cytotoxic genes, and antisense sequences as discussed above (*see also* U.S. Serial No. 08/032,846).

Cells may be removed from a variety of locations including, for example, from a selected tumor. In addition, within other embodiments of the invention, a vector construct may be inserted into non-tumorigenic cells, including for example, cells from the skin (dermal fibroblasts), or from the blood (*e.g.*, peripheral blood leukocytes). If desired, particular fractions of cells such as a T cell subset or stem cells may also be specifically removed from the blood (*see*, for example, PCT WO 91/16116, an application entitled "Immunoselection Device and Method"). Vector constructs may then be contacted with the removed cells utilizing any of the above-described techniques, followed by the return of the cells to the warm-blooded animal, preferably to or within the vicinity of a tumor. Within one embodiment of the present invention, subsequent to removing tumor cells from a warm-blooded animal, a single cell suspension may be generated by, for example, physical disruption or proteolytic digestion. In addition, division of the cells may be increased by addition of various factors such as melanocyte stimulating factor for melanomas or epidermal growth factor for breast carcinomas, in order to enhance uptake, genomic integration and expression of the recombinant viral vector.

Within the context of the present invention, it should be understood that the removed cells may not only be returned to the same animal, but may also be utilized to inhibit the growth of selected tumor cells in another, allogeneic, animal. In such a case it is generally preferable to have histocompatibility matched animals (although not always, *see, e.g.*, Yamamoto et al., "Efficacy of Experimental FIV Vaccines," 1st International Conference of FIV Researchers, University of California at Davis, September 1991).

The above-described methods may additionally comprise the steps of depleting fibroblasts or other non-contaminating tumor cells subsequent to removing tumor cells from a warm-blooded animal, and/or the step of inactivating the cells, for example, by irradiation.

5           As noted above, within certain aspects of the present invention, several anti-tumor agents may be administered either concurrently or sequentially, in order to inhibit the growth of a selected tumor in accordance with the methods of the present invention. For example, within one embodiment of the invention, an anti-tumor agent such as g-IFN may be co-administered or sequentially administered to a warm-blooded  
10 animal along with other anti-tumor agents such as IL-2, or IL-12, in order to inhibit or destroy a pathogenic agent. Such therapeutic compositions may be administered directly utilizing a single vector construct which directs the expression of at least two anti-tumor agents, or, within other embodiments, expressed from independent vector constructs. Alternatively, one anti-tumor agent (*e.g.*, g-IFN) may be administered  
15 utilizing a vector construct, while other tumor agents (*e.g.*, IL-2) are administered directly (*e.g.*, as a pharmaceutical composition intravenously).

          Within a particularly preferred embodiment, retroviral vector constructs which deliver and express both a g-IFN gene and another gene encoding IL-2, may be administered to the patient. In such constructs, one gene may be expressed from the  
20 retrovector LTR and the other may utilize an additional transcriptional promoter found between the LTRs, or may be expressed as a polycistronic mRNA, possibly utilizing an internal ribosome binding site. After *in vivo* gene transfer, the patient's immune system is activated due to the expression of g-IFN. Infiltration of the dying tumor with inflammatory cells, in turn, increases immune presentation and further improves the  
25 patient's immune response against the tumor.

          Within other aspects of the present invention, methods are provided for generating an immune response against an immunogenic portion of an antigen, in order to prevent or treat a disease (*see, e.g.*, U.S. Serial Nos. 08/104,424; 08/102,132, 07/948,358; 07/965,084), for suppressing graft rejection, (*see* U.S. Serial No.  
30 08/116,827), for suppressing an immune response (*see* U.S. Serial No. 08/116,828), and for suppressing an autoimmune response (*see* U.S. Serial No. 08/116,983).

          As will be understood by one of ordinary skill in the art given the disclosure provided herein, any of the retroviral vector constructs described herein may be delivered not only as a recombinant viral particle, but as direct nucleic acid vectors.  
35 Such vectors may be delivered utilizing any appropriate physical method of gene transfer, including for example, those which have been discussed above.

The following examples are offered by way of illustration, and not by way of limitation.

### EXAMPLE 1

#### CONSTRUCTION OF RETROVECTOR BACKBONES

##### A. Preparation of a Retroviral vector construct That Does Not Contain an Extended Packaging Sequence (Y)

5 This example describes the construction of a retroviral vector construct using site-specific mutagenesis. Within this example, a MoMLV retroviral vector construct is prepared wherein the packaging signal "Y" of the retrovector is terminated at basepair 617 of SEQ ID NO: 1, thereby eliminating the ATG start of *gag*. Thus, no  
10 crossover can occur between the retroviral vector construct and the *gag/pol* expression cassette which is described below in Example 3.

Briefly, pMLV-K (Miller, *J. Virol* 49:214-222, 1984 - an infectious clone derived from pMLV-1 Shinnick et al., *Nature*, 293:543-548, 1981) is digested with *Eco*57I, and a 1.9kb fragment is isolated. (*Eco*57I cuts upstream from the 3' LTR,  
15 thereby removing all *env* coding segments from the retroviral vector construct.) The fragment is then blunt ended with T4 polymerase (New England Biolabs), and all four deoxynucleotides, and cloned into the *Eco*RV site of phagemid pBluescript II KS+ (Stratagene, San Diego, Calif.). This procedure yields two constructs, designated pKS2+*Eco*57I-LTR(+) (Figure 1) and pKS2+*Eco*57I-LTR(-) (Figure 2), which are  
20 screened by restriction analysis. When the (+) single stranded phagemid is generated, the sense sequence of MoMLV is isolated.

A new *Eco*RI site is then created in construct pKS2+*Eco*57I-LTR(+) in order to remove the ATG start codon of *gag*. In particular, an *Eco*RI site is created using the single stranded mutagenesis method of Kunkle (*PNAS* 82:488, 1985).  
25 pKS2+*Eco*57I-LTR(+) is a pBluescript™ II + phagemid (Stratagene, San Diego, Calif.) containing an *Eco*57I fragment from pMLV-K. It includes the MoMLV LTR and downstream sequence to basepair 1378. When single stranded phagemid is generated the sense sequence of MoMLV is isolated. The oligonucleotide, 5'-GGT AAC AGT CTG GCC CGA ATT CTC AGA CAA ATA CAG (SEQ ID NO: 2), is created and  
30 used to generate an *Eco*RI site at basepairs 617-622. This construct is designated pKS2+LTR-*Eco*RI (Figure 3).

##### B. Substitution of Nonsense Codons in the Extended Packaging Sequence (Y+)

35 This example describes modification of the extended packaging signal (Y+) by site-specific mutagenesis. In particular, the modification will substitute a stop codon, TAA, at the normal ATG start site of *gag* (position 621-623 of SEQ ID NO: 1), and an additional stop codon TAG at position 637-639 of SEQ ID NO: 1.

Briefly, an *Eco57I* - *EcoRI* fragment (MoMLV basepairs 7770 to approx. 1040) from pN2 (Amentano et al., J. Virol. 61:1647-1650, 1987) is first cloned into pBluescript II KS+ phagemid at the *SacII* and *EcoRI* sites (compatible). Single stranded phagemid containing antisense MoMLV sequence, is generated using helper  
5 phage M13K07 (Stratagene, San Diego, Calif.). The oligonucleotide 5'-CTG TAT TTG TCT GAG AAT TAA GGC TAG ACT GTT ACC AC (SEQ ID NO: 3) is synthesized, and utilized according to the method of Kunkle as described above, in order to modify the sequence within the Y region to encode stop codons at nucleotides 621-623 and 637-639.

10

C. Removal of Retroviral Packaging Sequence Downstream from the 3' LTR

Retroviral packaging sequence which is downstream from the 3' LTR is deleted essentially as described below. Briefly, pKS2+*Eco57I*-LTR(-) (Figure 2) is  
15 digested with *BalI* and *HincII*, and relegated excluding the *BalI* to *HincII* DNA which contains the packaging region of MoMLV.

D. Construction of Vector Backbones

Constructs prepared in sections A and C above, or alternatively from  
20 sections B and C above, are combined with a plasmid vector as described below, in order to create a retrovector backbone containing all elements required *in cis*, and excluding all sequences of 8 nucleic acids or more contained in the retroviral portion of the *gag-pol* and *env* expression elements (see Examples 3 and 4).

1. Parts A and C are combined as follows: The product of A is  
25 digested with *NheI* and *EcoRI*, and a 1034 basepair fragment containing the LTR and minimal Y is isolated. The fragment is ligated into the product of part C at the unique (compatible) restriction sites *SpeI* and *EcoRI*. The resultant construct is designated pR1 (Figure 4)

2. Parts B and C are combined as follows: The product of B is  
30 digested with *NheI* and *EcoRI* and a 1456 basepair fragment containing the LTR and modified Y+ region is isolated. The fragment is ligated into the product of C at the unique (compatible) restriction sites *SpeI* and *EcoRI*. The resultant construct is designated pR2 (Figure 5).

35

## EXAMPLE 2

### INSERTION OF A GENE OF INTEREST INTO PR1 AND PR2

This example describes the insertion of a gene of interest, gp120, gp41, and rev along with a selectable marker into either pR1 or pR2. Briefly, the sequence encoding gp120, gp41 and rev is taken from construct pKT1 (Figure 6; *see also* Chada et al., *J. Vir.* 67:3409-3417, 1993); note that this vector is also referred to as N2IIIBenv. In particular, pKT1 is first digested at the unique *Asu*II site (position 5959). The ends are blunted, and an *Xho* I linker is ligated at that site. (New England Biolabs). The construct is then digested with *Xho* I, and a 4314 bp fragment containing HIV envelope (gp120 and gp41), rev, SV40 early promoter and G418 resistance genes is isolated.

pR1 or pR2 is digested at the unique *Eco* R1 restriction site, blunted, and *Sal* I linkers (New England Biolabs) are ligated in. The 4314 bp KT1 fragment is then ligated into pR1 or pR2 at the new *Sal* I sites, and the correct orientation is determined (*see* Figures 7 and 8). In both of these constructs, (pR1-HIVenv and pR2-IIIVenv) the HIV genes are expressed from the MLV LTR, and G418 resistance is expressed from the SV40 promoter.

## EXAMPLE 3

### CONSTRUCTION OF *GAG-POL* EXPRESSION CASSETTES

#### A. Construction of an Expression Cassette Backbone, pHCMU-PA

A vector is first created in order to form the backbone for both the *gag/pol* and *env* expression cassettes. Briefly, pBluescript SK- phagemid (Stratagene, San Diego, Calif.; GenBank accession number 52324; referred to as "SK-") is digested with *Spe*I and blunt ended with Klenow. A blunt end *Dra*I fragment of SV40 (Fiers et al., "Complete nucleotide sequence of SV40 DNA" *Nature* 273:113-120, 1978) from *Dra*I (bp 2366) to *Dra*I (bp2729) is then inserted into SK-, and a construct isolated in which the SV40 late polyadenylation signal is oriented opposite to the LacZ gene of SK-. This construct is designated SK-SV40A.

A Human Cytomegalovirus Major Immediate Early Promoter ("HCMV-IE"; Boshart et al., *Cell* 41:521-530, 1985) (*Hinc*II, bp 140, to *Eag*I, bp814) is isolated after digestion with *Hinc*II and *Eag*I, and the *Eag*I site blunt ended. The 674 blunt ended fragment is ligated into SK-SV40A. The final construct, designated pHCMV-PA is then isolated (*see* Figure 11). This construct contains the HCMV promoter oriented in opposite orientation to the LacZ gene, and upstream from the late polyadenylation signal of SV40.

### B. Creation of New Codons for the 5' Gag

This example describes *gag/pol* expression cassettes that lack non-coding sequences upstream from the *gag* start, thereby reducing recombination potential between the *gag-pol* expression element and Y+ sequence of a retroviral vector construct, and inhibiting co-packaging of the *gag-pol* expression element along with the retrovector. In order to construct such an expression cassette, 448 bp of DNA is synthesized with the following features: 5' ATATATATAT ATCGAT(*Clal* site) ACCATG(start codon, position 621) (SEQ ID NO: 4), followed by 410 bp encoding 136+ amino acid residues using alternative codons (*see* Figures 9 and 10), followed by GGCGCC(*NarI* site)AAACCTAAAC 3' (SEQ ID NO: 5).

Briefly, each of oligos 15 through 24 (set forth below in Table 1) are added to a PCR reaction tube such that the final concentration for each is 1  $\mu$ M. Oligos 25 and 26 are added to the tube such that the final concentration for each is 3  $\mu$ M. 1.2  $\mu$ L of 2.5 mM stock deoxynucleotide triphosphates (dG, dA, dT, dC) are added to the tube. 5  $\mu$ L of 10X PCR buffer (Perkin Elmer). Water is added to a final volume of 50  $\mu$ L. Wax beads are added and melted over the aqueous layer at 55°C and then cooled to 22°C. A top aqueous layer is added as follows: 5  $\mu$ L 10X PCR buffer, 7.5  $\mu$ L dimethylsulfoxide, 1.5  $\mu$ L Taq polymerase (Perkin-Elmer) and 36  $\mu$ L water. Forty cycles of PCR are then performed as follows: 94\_C, 30 seconds; 56\_C, 30 seconds; and 72\_C, 30 seconds. The PCR product is stored at -20\_C until assembly of the *gag/pol* expression cassette.

**Table 1**

SEQ. ID. No.	Sequence
15	5' ATA TAT ATA TAT CGA TAC CAT GGG GCA AAC CGT GAC TAC CCC TCT GTC CCT CA C ACT GGC CCA A 3'
16	5' TTG ATT ATG GGC AAT TCT TTC CAC GTC CTT CCA ATG GCC CAG TGT GAG GGA C 3'
17	5' AGA ATT GCC CAT AAT CAA AGC GTG GAC GTC AAA AAA CCG AGG TGG GT G ACA TTT TGT AGC GCC GAG TGG CCC 3'
18	5' AAG TTC CAT CCC TAG GCC AGC CAA CAT TGA ATG TGG GCC ACT CGG CGC TAC A 3'
19	5' GGC CTA GGG ATG GAA CTT TCA ATC GCG ATC TGA TTA CTC AAG TGA AA A TTA AAG TGT TCA GCC CCG GAC CCC 3'

- 20 5' GTG ACA ATA TAA GGA ACT TGA TCG GGA TGG CCG TGG GGT CCG GGG CTG  
AAC A 3'
- 21 5' AGT TCC TTA TAT TGT CAC ATC GGA GGC TCT CGC TTT CGA TCC ACC  
ACC TTG GGT GAA ACC ATT CGT GCA TCC 3'
- 22 5' AGG AGC GCT GGG TGG GAG GGG TGG AGG TGG TTT GGG ATG CAC GAA  
TGG TTT C 3'
- 23 5' CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC  
ACA CCA CCC AGG AGC AGC TTG TAC CCT 3'
- 24 5' GTT TAG GTT TGG CGC CGA GGC TGG GGG TCA GAG CAG GGT ACA AGC TGC  
TCC T 3'
- 25 5' ATA TAT ATA TAT CGA TAC C 3'
- 26 5' GTT TAG GTT TGG CGC CGA GG 3'

### C. Creation of a New 3' End for *Pol*

In order to prepare a *gag/pol* expression cassette which expresses full length *gag/pol*, pCMV*gag/pol* is constructed. Briefly, MoMLV sequence from *Pst*I (BP567) to *Nhe*I (bp 7847) is cloned into the *Pst*I-*Xba*I sites of pUC19 (New England Biolabs). The resultant intermediate is digested with *Hind*III and *Xho*I, and a 1008 bp fragment containing the *gag* leader sequence is isolated. The same intermediate is also digested with *Xho*I and *Sca*I, and a 4312 bp fragment containing the remaining *gag* and *pol* sequences is isolated. The two isolated fragments are then cloned into the *Hind*III and *Sma*I sites of pHCMV-PA, described above. The resultant construct, designated CMV *gag/pol* (Figure 12) expresses MoMLV *gag* and *pol* genes.

In order to truncate the 3' end of the *pol* gene found in pCMV *gag-pol*, a 5531 basepair *Sna*BI - *Xma*I fragment containing a portion of the CMV IE promoter and all of *gag-pol* except the final 28 codons, is isolated from pCMV *gag-pol*. This fragment is cloned into the *Sna*BI and *Xma*I sites of pHCMV-PA. This construct expresses five new amino acids at the carboxy-terminus (Ser-Lys-Asn-Tyr-Pro) (SEQ ID NO: 6) (pCMV gpSma).

Alternatively, these five amino acids may be eliminated by digesting pCMVgp *Sma*I with *Sma*I and adding an *Nhe*I (termination codons in three phases) linker (5' - CTA GCT AGC TAG) (SEQ ID NO: 14; New England Biolabs) at the end of the truncated *pol* sequence. This construct is designated pCMV gp *Nhe*. Both of these constructs eliminates potential crossover between *gag/pol* and *env* expression cassettes.



D. Gag-Pol Expression Cassette

Parts B and C from above are combined to provide an expression vector containing a CMV IE promoter, *gag-pol* sequence starting from the new *Clal* site (followed by ACC ATG and 412 bp of alternative or "wobble" *gag* coding sequence) and terminating at the *SmaI* site (MoMLV position 5750) followed by an SV40 polyadenylation signal, essentially as described below. Briefly, the approximately 451 bp double stranded wobble fragment from part A is ligated into pCR'II TA cloning vector (Invitrogen Corp.). The wobble PCR product naturally contains a 3' A-overhang at each end, allowing for cloning into the 3' T-overhang of pCR'II. The 422 bp *Clal* - *NarI* wobble fragment from the pCR'II clone is removed and is ligated into the *Clal* (Position 679, Figure pCMV gp *Sma*) and *NarI* (Position 1585) sites of pCMVgp *SmaI* (Part B) (or pCMV gp *Nhe*). (The *Clal* site at position 5114 is methylated and not cut with *Clal*). The product of that ligation is digested with *NarI*, and the MLV-K *NarI* fragment (positions 1035 to 1378) is inserted (SEQ ID NO: 1). This construct is designated pCMVgp -X (Figure 14).

EXAMPLE 4

CONSTRUCTION OF *ENV* EXPRESSION CASSETTES

A. Creation of a New 5' *EagI* Restriction Site

Starting with an *EagI*- *EcoRI* 626 bp subfragment from a 4070A amphotropic envelope (Chattopodhyay et al., *J. Vir.* 39:777, 1981; GenBank accession # MLV4070A, and #MLVENVC; SEQ ID NO: 13) cloned in a pBluescript II Ks+ vector (containing the start codon), site directed mutagenesis is performed upstream of the translation start site in order to change ACCATCCTCT GGACGGACAT G... (SEQ ID NO: 7; positions 19 - 39 of Genebank sequence # MLVENVC) to ACCCGGCCGT GGACGGACAT G... (SEQ ID NO: 8) and create a new *EagI* site at position 23. This modification allows cloning of the amphotropic envelope sequence into an expression vector eliminating upstream 4070A sequence homologous to the *gag-pol* expression element as described in Example 2A.

B. Creation of a New 3' End for Env

A new 3' end of the envelope expression element is created by terminating the sequence which encodes the R-peptide downstream from the end of the transmembrane region (p15E). Briefly, construct pHCMV-PA, described above, is first modified by digestion with *NotI* (position 1097), blunted and relegated to obliterate the overlapping Bluescript *EagI* site at the same position. pCMV Envam-Eag-X-less is

then constructed by digesting the modified pHCMV-PA with *EagI* (position 671 and *SmaI* (position 712) and ligating in two fragments. The first is an *EagI-NcoI* fragment from 4070A (positions 1-1455) (SEQ ID NO: 13). The second is an MLV-K envelope fragment, *NcoI* - *PvuII* (positions 7227-7747) (SEQ ID NO: 1). The resultant construct from the three-way ligation contains the HCMV promoter followed by the SU (GP70) coding sequence of the 4070A envelope, the TM (p15e) coding sequence of MoMLV, and sequence encoding 8 residues of the R-peptide. In addition, this envelope expression cassette (pCMV Env am-Eag-X-less) (Figure 18) shares no sequence with crossless retrovector backbones described in Example 1.

### C. Envelope Expression Element

Parts A and B from above are combined to complete an amphotropic expression element containing the CMV promoter, 4070A SU, MoMLV TM and SV40 polyadenylation signal in a Bluescript SK- plasmid vector. This construct is called pCMVenv-X (Figure 15). Briefly, the construct described in part A with a new *EagI* restriction site is digested with *EagI* and *XhoI*, and a 571 bp fragment is isolated. pCMV Envam-Eag-X-less (from part B) is digested with *KpnI* and *EagI* and the 695 bp fragment is reserved. pCMV Envam-Eag-X-less (from part B) is digested with *KpnI* and *XhoI* and the 4649 bp fragment is reserved. These two fragments are ligated together along with the 571 bp *EagI* to *XhoI* fragment digested from the PCR construct from part A. pCMVenv-X shares no sequence with crossless retrovector backbones nor the *gag-pol* expression element pCMVgp-X.

## EXAMPLE 5

### FUNCTIONALITY TESTS FOR *GAG-POL* AND *ENV* EXPRESSION CASSETTES

Rapid tests have been developed in order to ensure that the *gag-pol* and *env* expression cassettes are biologically active. The materials for these tests consist of a cell line used for transient expression (typically 293 cells, ATCC #CRL 1573), a target cell line sensitive to infection (typically HT 1080 cells, ATCC #CCL 121) and either pRgpNeo (Figure 16) or pLARNL (Emi et al., *J. Virol* 65:1202-1207, 1991). The two later plasmids express rescuable retrovectors that confer G418 resistance and also express *gag-pol*, in the case of RgpNeo or *env*, in the case of pLARNL. For convenience, the organization of RgpNeo (Figure 16) is set forth below.

In order to test expression cassettes such as pCMVgp-X for functionality of *gag/pol*, the plasmid is co-transfected with pLARNL at a 1:1 ratio into 293 cells. After 12 hours, the media is replaced with normal growth media. After an additional 24

hours, supernatant fluid is removed from the 293 cells, filtered through a 0.45 mm filter, and placed on HT 1080 target cells. Twenty-four hours after that treatment, the media is replaced with growth media containing 800 µg/ml G418. G418 resistant colonies are scored after one week. The positive appearance of colonies indicates that all elements  
5 are functional and active in the original co-transfection.

For convenience, the organization of RgpNeo (Figure 16) is set forth below:

Position 1 = left end of 5' LTR; Positions 1-6320 = MoMLV sequence from 5'LTR to Sca 1 restriction site; Positions 6321 - 6675 = SV40 early promoter; Positions 6676-  
10 8001 = Neo resistance gene from Tn 5 (including prokaryotic promoter); and Positions 8002 - 8606 = pBR origin of replication.

### EXAMPLE 6

#### PACKAGING CELL LINE AND PRODUCER CELL LINE DEVELOPMENT

15

This example describes the production of packing and producer cell lines utilizing the above described retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes, which preclude the formation of replication competent virus.

20

Briefly, for amphotropic MoMLV-based retroviral vector constructs, a parent cell line is selected which lacks sequences which are homologous to Murine Leukemia Viruses, such as the dog cell line D-17 (ATCC No. CCL 183). The *gag/pol* expression cassettes are then introduced into the cell by electroporation, along with a selectable marker plasmid such as DHFR (Simonsen et al., *PNAS* 80:2495-2499, 1983).  
25 Resistant colonies are then selected, expanded in 6 well plates to confluency, and assayed for expression of *gag/pol* by Western Blots. Clones are also screened for the production of high titer vector particles after transduction with pLARNL.

25

The highest titer clones are then electroporated with an *env* expression cassette and a selectable marker plasmid such as hygromycin (*see* Gritz and Davies,  
30 *Gene* 25:179-188, 1983). Resistant colonies are selected, expanded in 6 well plates to confluency, and assayed for expression of *env* by Western Blots. Clones are also screened for the production of high titer vector particles after transduction with a retroviral vector construct.

Resultant packaging cell lines may be stored in liquid Nitrogen at 10 x  
35 10<sup>6</sup> cells per vial, in DMEM containing 10% irradiated Fetal Bovine Serum, and 8% DMSO. Further testing may be accomplished in order to confirm sterility, and lack of

helper virus production. Preferably, both an S+L- assay and a *Mus dunni* marker rescue assay should be performed in order to confirm a lack of helper virus production.

In order to construct a producer cell line, retroviral vector construct as described above in Example 1 is electroporated into a xenotropic packaging cell line made utilizing the methods described above. After 24-48 hours, supernatant fluid is removed from the xenotropic packaging cell line, and utilized to transduce a second packaging cell line, thereby creating the final producer cell line.

#### EXAMPLE 7

##### HELPER DETECTION ASSAY COCULTIVATION, AND MARKER RESCUE

This example describes a sensitive assay for the detection of replication competent retrovirus ("RCR"). Briefly,  $5 \times 10^5$  vector-producing cells are cocultivated with an equal number of *Mus dunni* cells (Lander and Chattopadhyay, *J. Virol.* 52:695, 1984). *Mus dunni* cells are particularly preferred for helper virus detection because they are sensitive to nearly all murine leukemia-related viruses, and contain no known endogenous viruses. At three, six, and nine days after the initial culture, the cells are split approximately 1 to 10, and  $5 \times 10^5$  fresh *Mus dunni* cells are added. Fifteen days after the initial cocultivation of *Mus dunni* cells with the vector-producing cells, supernatant fluid is removed from cultures, filtered through a 0.45 mm filter, and subjected to a marker rescue assay.

Briefly, culture fluid is removed from a MdH tester cell line (*Mus dunni* cells containing pLHL (a hygromycin resistance marker retroviral vector; see Palmer et al., *PNAS* 84(4):1055-1059, 1987) and replaced with the culture fluid to be tested. Polybrene is added to a final concentration of 4 mg/ml. On day 2, medium is removed and replaced with 2 ml of fresh DMEM containing 10% Fetal Calf Serum. On day 3, supernatant fluid is removed, filtered, and transferred to HT1080 cells. Polybrene is added to a final concentration of 4mg/ml. On day 4, medium in the HT1080 cells is replaced with fresh DMEM containing 10% Fetal Calf Serum, and 100 mg/ml hygromycin. Selection is continued on days 5 through 20 until hygromycin resistant colonies can be scored, and all negative controls (e.g., mock infected MdH cells) are dead.

### EXAMPLE 8

#### ASSAY FOR ENCAPSIDATION OF WOBBLE RNA SEQUENCE

This example describes a sensitive assay for the detection of encapsidation of RNA from constructs containing wobble or normal gag sequence. Briefly, a fragment of DNA from a "wobble" *gag/pol* expression cassette (Example 3), containing the CMV promoter and gag sequence to the *Xho*I site (MoMLV position 1561) is ligated to a SV40 neo-3' LTR DNA fragment from N2 (Armentano et al., *supra*) or KT-3 (*see* WO 91/02805 or WO 92/05266). This construct is diagrammatically illustrated in Figure 19A, and is not expected to be encapsidated in packaging cell lines such as DA or HX (*see* WO 92/05266) because it lacks a 5' LTR and primer binding site.

A second construct is also made, similar to the first except that the wobble sequence is replaced by normal *gag* sequence. Similar to the first construct, the RNA transcribed from this DNA is not expected to be encapsidated. This construct is diagrammatically illustrated in Figure 19B.

The above constructs are separately transfected into a packaging cell line. The culture is then assayed for the ability to generate transducible G418-resistant retrovector. Neither construct results in transducible vector.

Cell cultures containing the above constructs are then transduced with the retrovector LHL (*see* Example 7). The cell cultures, after selection, will now generate retrovector conferring hygromycin resistance to target cells. Further, if co-encapsidation is allowed by interaction between LHL RNA and the transcripts from the above constructs, statistically significant RT-mediated recombination can occur resulting in the transfer of G418 resistance to target cells.

### EXAMPLE 9

#### CONSTRUCTION OF RETROVIRAL BACKBONES

This example describes several modifications of the retroviral vector pKT1 (Figure 6) resulting in decreased sequence homology to the retroviral *gag/pol* and envelope expression constructs. In addition, two stop codons were introduced in the DNA sequence of the packaging signal sequence in order to increase the safety of these vectors. All modifications are summarized in Fig. 20 and the resulting retroviral backbone is called pBA-5b.

A. Substitution of Nonsense Codons in the Extended Packaging Sequence ( $\Psi^+$ )

This example describes modification of the extended packaging signal ( $\Psi^+$ ) by PCR on the template KT-1 using primers that introduce two stop codons in the extended packaging signal sequence. -In particular, the template pKT-1 contains the modification ATT at the normal ATG start site of *gag* (position 621-623 of SEQ ID NO: 1). Here the start site was further modified to the stop codon, TAA, and an additional stop codon TGA was added to replace the codon TTA at position 645-647 of SEQ ID NO: 1.

10 Briefly, two sets of PCR reactions were carried out on pKT1, each introducing one stop codon. The primers for the PCR were designed such that the two PCR products had overlapping regions and a splice-overlap extension PCR (SOE-PCR) was carried out with the two PCR products in order to combine the two introduced stop codons on one strand. The first set of oligonucleotides introducing the change from  
15 ATT to TAA were 5'-GGG-AGT-GGT-AAC-AGT-CTG-GCC-TTA-ATT-CTC-AG (SEQ ID NO: 27) and 5'-CGG-TCG-ACC-TCG-AGA-ATT-AAT-TC (SEQ ID NO: 28) and the second set of oligonucleotides introducing the change from TTA to TGA were 5'-CTG-GGA-GAC-GTC-CCA-GGG-ACT-TC (SEQ ID NO: 29) and 5'-GGC-CAG-ACT-GTT-ACC-ACT-CCC-TGA-AGT-TTG-AC (SEQ ID NO: 30). The flanking  
20 primers of the final 708 base pair PCR product introduced the *AatII* and the *XhoI* sites, at the 5' and 3', respectively.

The ends of the 708 base pair product were blunted and phosphorylated and the product introduced into the *SmaI* and *EcoRV* digested vector pBluescript SK- (Stratagene, San Diego, Calif.). The resulting plasmid was designated pBA-2, and is  
25 shown diagrammatically in Figure 20.

B. Removal of Retroviral Sequences Upstream and Downstream from the 3' LTR and Upstream and within the 5' LTR

30 Retroviral envelope sequence was removed upstream of the 3' LTR between the *Clal* site and the TAG stop codon of the envelope coding sequence. The DNA sequence was modified by PCR such that the TAG stop codon was replaced by a *Clal* site and the 97 nucleotides upstream from this new *Clal* site to the original *Clal* site were deleted, as well as the 212 base pairs of retroviral sequence downstream of the 3' LTR.

35 Briefly, the following two oligonucleotides were used for the PCR: 5'-CATCGATAAA ATAAAAGATT TTATTAGTC (SEQ ID NO: 31) and 5'-CAAATGAAAG ACCCCCGCTG AC (SEQ ID NO: 32) and the template was pKT1.

The PCR product was cloned into pPCRII (Invitrogen, San Diego, Calif.) using the TA cloning kit (Invitrogen, San Diego, Calif.) and called pBA-1.

Subsequently, pBA-2 (described in section A above) was digested with *XbaI* and *AatII* which deleted a part of the multiple cloning site and into this linearized  
5 vector the 780 base pair fragment from *NheI* to *AatII* from pKT1 was cloned, resulting in the plasmid pBA-3. This plasmid pBA-3 combined the shortened 5' LTR with the above described packaging region including the two introduced stop codons.

Subsequently, pBA-1 was digested with *Clal* and *Apal* resulting in a 640  
10 base pair fragment that was cloned into the *Clal* and *Apal* digested pBA-3 resulting in the plasmid pBA-4. This plasmid combines the above described 5' LTR and the packaging signal with the 3' LTR.

Subsequently, pBA-4 was digested with *Apal* and *EcoRI*, ends blunted and religated in order to remove extraneous 3' polylinker sites, resulting in plasmid pBA-5a.

Subsequently, pBA-5a was cut with *NotI* (blunted) and *EcoRI* and  
15 introduced into *SmaI* and *EcoRI* digested pUC18 (GIBCO/BRL, Gaithersburg, MD) resulting in pBA-5b. This construct moved the retroviral vector from a pBluescript into an alternate pUC18 vector.

20

### EXAMPLE 10

#### INSERTION OF GENES OF INTEREST INTO CROSS-LESS RETROVIRAL VECTOR BACKBONE PBA-5B

This example describes the insertion of two genes of interest,  
25 gp120(HIVenv/rev) and HSV-TK along with the neomycin gene into pBA-5b. Briefly, the sequence encoding gp120 was taken from construct pKT1 (Figure 6; see also Chada et al., *J. Vir.* 67:3409-3417, 1993). This vector is also referred to as N2IIIBenv.

#### A. Introduction of HSV-TK and Neomycin into the Retroviral Vector pBA-5b

30

The HSV-TK gene was retrieved by digesting pBH-1 (PCT#UU 091-02805) with *XhoI* and *EcoRI* resulting in a 1.2 kb fragment. The neomycin gene driven by the SV40 promoter was retrieved by digesting pKT1 with *EcoRI* and *BstBI* resulting in a 1.3 kb fragment. Both fragments were cloned into a *XhoI* and *Clal* digested pBA-  
35 5b resulting in the retroviral vector pMO-TK.

B. Introduction of HIVenv/rev and neomycin into the retroviral vector pBA-5b

The HIVenv/rev and neomycin genes were retrieved by digesting pKT1 with *Xho*I and *Bst*BI resulting in a 4.4 kb fragment which was cloned into the *Xho*I and  
5 *Cl*AI digested pBA-5b resulting in the retroviral vector pBA-6b.

EXAMPLE 11

FUNCTIONALITY TESTS FOR THE CROSS-LESS RETROVIRAL BACKBONES  
pBA-6B AND PMO-TK

10

Rapid tests are described in more detail below which ensure that the retroviral vectors coding for HIVenv/rev and neomycin (pBA-6b) and HSV-Tk and neomycin (PMO-TK) are comparable to the original retroviral vectors with regard to expression levels the genes of interest (HIVenv/rev and HSV-TK) and titers.

15

A. Comparison of Transient and Stable Neo titer from pKT1 Versus pBA-6 in Transfected Non-clonal Vector Producing Pools

Retroviral vectors pKT1 or pBA-6 were transfected into DA packaging cells via  $\text{CaPO}_4$ -precipitation using the ProFection kit from Promega according to  
20 manufacturer's protocol (Promega, Madison, WI). The transient supernatant was collected 48 hours posttransfection, sterile-filtered (0.45  $\mu\text{m}$ ) and placed on HT 1080 target cells (HT 1080 cells, ATCC #CCL 121) in the presence of 8 mg polybrene/ml. Twenty-four hours after that treatment, the media is replaced with growth media containing 800  $\mu\text{g/ml}$  G418. G418 resistant colonies are scored after one week. The  
25 positive appearance of colonies indicates that all elements are functional and active in the original co-transfection.

For the stable neo titer, the transfected DA cells were cultured in selection media containing 800  $\mu\text{g/ml}$  G418 for two weeks or until the untransfected control cells were dead. Titer of the supernatants from the confluent vector producing  
30 pools was determined as described above.



Results of transient and stable neo titers are presented in Table 2.

Table 2:

- 5 Transient and stable neo titer of pKT-1 versus pBA-6b retroviral vectors in transfected and selected non-clonal DA vector producing pools.

Retroviral vector coding for HIV env/rev plus neo	Transient neo titer in CFU/ml
pKT-1	$5.0 \times 10^4$
pBA-6b (cross-less retroviral vector)	$2.5 \times 10^4$
	Stable neo titer in CFU/ml
pKT-1	$5.6 \times 10^6$
pBA-6b (cross-less retroviral vector)	$6.7 \times 10^6$

- 10 B. Comparison of gp120 and rev expression levels of pKT1 versus pBA-6b in vector producing pools and target cells.

The supernatants from the above described selected non-clonal pools DA/KT1 and DA/BA-6b were used to transduced HT 1080 target cells as described above. G-418 resistant colonies were selected as described above and the pools were named HT 1080/KT1 and HT 1080/BA-6b.

- 15 The DA/KT1, DA/BA-6b vector producer pools as well as the HT 1080/KT1 and HT 1080/BA-6b pools were lysed and gp120 and rev protein levels were estimated by Western Blot analysis according to standard procedures.

Results are presented in Table 3.

Table 3:

- 5 Comparison of pKT1 and pBA-6b retroviral vector with regard to gp120 and rev expression levels in transduced and selected non-clonal DA vector producing pools and transduced and selected target cells.

G-418 selected pools analyzed	gp120 expression levels	Rev expression levels
DA	-	-
DA/KT1	+	+
DA/BA-6b	++	+
HT-1080	-	-
HT-1080/KT1	+++	+
HT-1080/BA-6b	+++	+

- 10 C. Comparison of Stable Neo Titer from pKT1 Versus pBA-6 in Transduced Non-Clonal Vector Producing Pools

The retroviral vectors pBH1 or pMO-TK were transduced into various packaging cell lines using transient transfection produced VSV-G pseudotyped vectors as described in PCT/US91/06852 entitled "Packaging Cells" and PCT/US91/05699  
 15 entitled "Viral Particles Having Altered Host Range." The following packaging cell lines were used: DA, HA, HP, HX, 2A, 2X, as described in PCT/US91/06852 and PCT/US91/05699.

For the stable neo titer, the transduced packaging cell line pools were cultured in selection media containing 800 µg/ml G418 for two weeks or until the  
 20 untransfected control cells were dead. Titers of the supernatants from the confluent vector producing pools were determined as described above.

TK expression levels were determined by Western Blot analysis of lysates of the specified vector producing pools.

- 25 Results of stable neo titers as well as TK expression levels in the various vector producing pools are presented in Table 4.

Table 4:

5 Comparison of pBH-1 and pMO-TK in various packaging cell lines with regard to neo titers and TK expression levels in the transduced and selected vector producing pools.

G-418 selected pools analyzed	Stable neo titers (CFU/ml)	TK expression levels
DA/BH-1	$6.0 \times 10^5$	++
DA/MO-TK	$1.3 \times 10^6$	++
HA/BH-1	$3.7 \times 10^5$	+++
HA/MO-TK	$1.6 \times 10^5$	+++
HP/BH-1	$< 1 \times 10^3$	++
HP/MO-TK	$< 1 \times 10^3$	++
HX/BH-1	$3.5 \times 10^5$	++
HX/MO-TK	$1.0 \times 10^5$	++
2A/BH-1	$1.3 \times 10^5$	+
2A/MO-TK	$1.7 \times 10^5$	+
2X/BH-1	$3.2 \times 10^5$	+
2X/MO-TK	$5.2 \times 10^5$	+

### EXAMPLE 12

#### CONSTRUCTION OF *ENV* EXPRESSION CASSETTES

10

##### A. Cloning of Long and Short Bovine Growth Hormone Termination-Polyadenylation Sequences

The Long Bovine Growth Hormone (BGH) termination-polyadenylation sequence (positions 2330-2551 of Genebank sequence # BOVGHHG) was PCR  
 15 amplified from the plasmid pCDNA3 (Invitrogen Corp, San Diego CA) using the forward primer 5'CCTATGAGCT CGCCTTCTAG TTGCCAGC (SEQ ID NO: 33) (positions 2330-2346 of Genebank sequence # BOVGHHG) containing a restriction site for *Sac* I restriction endonuclease (underlined) and the reverse primer 5'CCTATGAATT CGCGGCCGCC ATAGAGCCCA CCGCATCC (SEQ ID NO: 34)  
 20 (positions 2551-2531 of Genebank sequence # BOVGHHG) containing restriction sites for *Eco*R I and Not I (underlined). The PCR fragment was digested with *Sac* I and *Eco*R

I and inserted into *Sac* I/*Eco*R I digested pBGS131 vector (American Type Culture Collection #37443) to create pBGS-long BGH. Similarly, the short BGH termination-polyadenylation sequence (positions 2415-2463 of Genebank sequence # BOVGHHG) was PCR amplified using the forward primer 5'TATATATGAG CTCTAATAAA  
 5 ATGAGGAAAT TGCATCGCAT TGTC (SEQ ID NO: 35) (positions 2415-2445 of Genebank sequence # BOVGHHG) containing a restriction site for *Sac* I restriction endonuclease (underlined) and the reverse primer 5'CCTATGAATT CGCGGCCGCA  
 TAGAATGACA CCTACTCAGA CAATGCGA (SEQ ID NO: 36) (positions 2463-2436 of Genebank sequence # BOVGHHG) containing the restriction sites for *Eco*R I  
 10 and *Not* I (underlined). A template was not required because the primer sequences overlap. The PCR fragment was digested with *Sac* I and *Eco*R I and inserted into *Sac* I/*Eco*R I digested pBGS131 vector to create pBGS-short BGH.

#### B. Creation of a New 3' End for Env

15 The entire MoMLV amphotropic envelope coding region was PCR amplified from the plasmid pCMVenvAmDra (described in PCT/US91/06852 example 2) using the forward primer GCTCGTTTAG TGAACCGTCA G (SEQ ID NO: 37) (positions 606-631 of pCMVenvAmDra) and the reverse primer TATCCGAGCT  
 20 CATGGCTCGT ACTCTATGG (SEQ ID NO: 38) (positions 3136-3118 of pCMVenvAmDra). The reverse primer contains the restriction site for *Sac* I restriction endonuclease (underlined) directly after the stop codon of amphotropic envelope (bold). The PCR fragment was digested with *Sac* I and *Bgl* II and inserted into *Sac* I/*Bgl* II digested pBGS-long BGH and pBGS-short BGH vector to create pBGSAmEnv-long BGH and pBGSAmENV-short BGH respectively. These constructs contain  
 25 amphotropic envelope with no MoMLV sequence past the termination codon, followed by the BGH termination-polyadenylation sequence.

#### C. Insertion of Env-BGH into an Expression Plasmid

The plasmid pCMVenvAmDra (described in PCT/US91/06852, Example  
 30 2) was digested with *Bst*B I and *Not* I restriction endonucleases. This digest removes approximately 210 bases of the 3' coding region of amphotropic envelope, approximately 75 MoMLV 3'noncoding bases, and the SV40 termination-polyadenylation sequence. The small *Bst*B I/*Not* I fragment of the plasmids pBGSAmEnv-long BGH and pBGSAmENV-short BGH was inserted into the *Bst*B  
 35 I/*Not* I digested pCMVenvAmDra expression plasmid to create pCMVenvAmDra/LBGH and pCMVenvAmDra/SBGH respectively. The small *Bst*B I/*Not* I fragment of the plasmids pBGSAmEnv-long BGH and pBGSAmENV-short

BGH was also inserted into *Bst*B I/*Not* I digested plasmid pCMVenv-X (Example 4) to create the plasmids pCMVenvX-long BGH and plasmids pCMVenvX-short BGH.

5       D.     Construction of the envelope gene truncated in the 5' and 3' non-coding regions of pCI

          The entire MoMLV amphotropic envelope coding region was PCR amplified from the plasmid pCMVenvAmDra (described in PCT/US91/06852, Example 2) using the forward primer 5' CACCTATGCT AGCCACCATG GCGCGTTCAA CGCTCTC (SEQ ID NO: 39) containing a restriction site for *Nhe*I restriction  
10    endonuclease (underlined) and the reverse primer 5' CACCTATGCG GCCGCTCATG GCTCGTACTC TATGGG (SEQ ID NO: 40) containing a restriction site for *Not*I restriction endonuclease (underlined). The PCR fragment was digested with *Not*I and *Nhe*I and inserted into a *Not*I/*Nhe*I digested pCI vector (Promega Corp, Madison WI) to create pCI/envam. The PCR fragment contains the entire coding region of the envelope  
15    gene including the *Nhe*I site followed by the Kozak sequence CACC upstream of the ATG start codon and the TCA stop codon followed by the *Not*I site.

          E.     Construction of the envelope gene truncated in the 5' and 3' non-coding regions in pCMVb

20       Similarly to pCI/envam, the entire MoMLV amphotropic envelope coding region was PCR amplified from the plasmid pCMVenvAmDra (described in PCT/US91/06852, Example 2) using the forward primer 5' CACCTATGCG GCCGCCACCA TGGCGCGTTC AACGCTCTC (SEQ ID NO: 41) containing a  
restriction site for *Not*I restriction endonuclease (underlined) and the reverse primer 5'  
25    CACCTATGCG GCCGCTCATG GCTCGTACTC TATGGG (SEQ ID NO: 40) containing a restriction site for *Not*I restriction endonuclease (underlined). The PCR fragment was digested with *Not*I and inserted into the *Not*I digested pCMVb vector (Clontech Laboratories Inc., Palo Alto, CA) deleting the b-galactosidase gene from pCMVb to create pCMV-b/envam. The PCR fragment contains the entire coding region  
30    of the envelope gene including the *Not*I site followed by the Kozak sequence CACC upstream of the ATG start codon and the TCA stop codon followed by the *Not*I site.

          F.     Construction of the envelope gene truncated in the 5' and 3' non-coding regions in pCMVenvAmDra/LBGH/ EAG del.

35       The plasmid pCMVenvAmDra/LBGH/EAG del was constructed from the plasmid pCMVenvAmDra/LBGH (described in example 12-c) by deletion of 441 base pairs from the *ag*I site at position 695 to the *Eag*I site at position at 1136 just upstream of the env start codon. This was accomplished by digesting

pCMVenvAmDra/LBGH with *agI* and gel purifying the resulting bands of 2,227 and 3,573 base pairs. These two fragments were then ligated together and screened for correct orientation, such that the env start site was positioned downstream of the CMV promoter. The resulting construct was named pCMVenvAmDra/LBGH/EAG del.

5

### EXAMPLE 13

#### CONSTRUCTION OF VARIOUS *GAG/POL* EXPRESSION PLASMIDS WITH PARTIALLY OR COMPLETELY REDUCED SEQUENCE OVERLAP TO THE CROSS-LESS RETROVIRAL BACKBONE AND ENVELOPE

10

This example describes several modifications of the MoMLV *gag/pol* expression plasmid pSCV10 (PCT/US91/06852, WO 92/05266) resulting in decreased or eliminated sequence homology to the retroviral backbone and envelope expression constructs.

15

##### A. Creation of New Codons for the 5'Gag

This example describes the *gag/pol* expression plasmid cassette that contains wobbled non-coding sequences upstream from the *gag* start site, thereby reducing recombination potential between the *gag/pol* expression element and the extended packaging signal of a retroviral vector construct, and inhibiting co-packaging of the *gag/pol* expression element along with the retrovector. In order to construct such an expression cassette a 406 bp DNA fragment with a *Clal* site at the 5' end (underlined) and a *NarI* site at the 3' end (underlined) was synthesized by Operon (Operon Technologies Inc, Alameda CA). The sequence of the 406 bp DNA fragment was verified and is provided in Table 5. The synthesized DNA was transferred to a shuttle plasmid as a *Clal-NarI* fragment to create the plasmid pWOB.

Table 5

30 ATCGATACCCATGGGGCAAACCGTGACTACCCCTCTGTCCCTCACACTGGGCC  
ATTGGAAGGACGTGGAAAGAATTGCCCATTAATCAAAGCGTGGACGTCAAAA  
AACGCAGGTGGGTGACATTTTGTAGCGCCGAGTGGCCACATTCAATGTTG  
GCTGGCCTAGGGATGGAACCTTCAATCGCGATCTGATTACTCAAGTGAAAA  
TTAAAGTGTTACGCCCCGGACCCACGGCCATCCCGATCAAGTTCCTTATAT  
35 TGTACATGGGAGGCTCTCGCTTTCGATCCACCACCTTGGGTGAAACCATTC  
GTGCATCCCAAACACCTCCACCCCTCCACCCAGCGCTCCTAGCCTGCCCT

TGGAGCCCCCACGAAGCACACCACCCAGGAGCAGCTTGTACCCTGCTCTGA  
CCCCCAGCCTCGGGCGCC (SEQ ID NO: 42)

The *ClaI-NarI* fragment from pWOB was isolated by *ClaI-NarI* digest, and the 406 bp  
5 fragment cloned into the *ClaI-NarI* site of pSCV10 to create the plasmid pSCV10/wob  
(-NarI fragment) which resulted in the loss of the 481 bp *NarI-NarI* fragment just  
downstream of the wobbled *ClaI-NarI* fragment.

10 B. Creation of a 5' truncated *gag/pol* construct without MoMLV sequence  
upstream of the start codon in pSCV10

This example describes the *gag/pol* expression plasmid cassette that  
eliminated the MoMLV sequence upstream of the ATG start codon in order to prevent  
sequence overlap to the retroviral backbone.

Briefly, a new *ClaI* site followed by an ideal Kozak translational start  
15 sequence was introduced upstream of the start codon of the *gag/pol* construct pSCV10  
by PCR using the forward primer 5' CGAATCGATA CCATGGGCCA  
GACTGTTACC AC (SEQ ID NO: 43) (the *ClaI* site is underlined) and the reverse  
primer 5' CATTCTGCAG AGCAGAAGGT AAC (SEQ ID NO: 44) containing a  
restriction site for *PstI* (underlined). The PCR fragment was digested with *ClaI* and  
20 *PstI* and the 131 bp fragment cloned into pSCV10 replacing the existing *ClaI-PstI* DNA  
fragment to create the plasmid pSCV10/5'truncated g/p.

25 C. Creation of a 5' truncated *gag/pol* construct without MoMLV sequence  
upstream of the start codon in pCI

This example describes the construction of the 5' truncated *gag/pol*  
construct analogous to that described under section B above in the pCI (Promega Corp,  
Madison, WI) vector backbone.

Briefly, fragments were prepared for a three-way ligation as follows:  
pCI was digested with *SmaI* and *NotI* and the 4 kb fragment was isolated. pSCV10 was  
30 digested with *XhoI* and *NotI* and the 4.7 kb fragment was isolated. pSCV10/5'  
truncated g/p as described in section B was digested with *ClaI*, filled in with Klenow to  
blunt, then digested with *XhoI* and the 0.95 kb fragment was isolated. These three  
fragments were then ligated together to give the final plasmid pCI/5'truncated g/p.

35 D. Creation of a 5' truncated and wobbled *gag/pol* construct in pCI

This example describes the construction of the 5' truncated and wobbled  
*gag/pol* construct in the pCI vector where the 5' truncation as described in section C  
and the wobbled *gag* sequences as described in section A were combined.

Briefly, the wobbled *gag/pol* sequence (0.47 kb) was retrieved from plasmid pSCV10/wob (-*NarI* fragment) as described in section A above by digestion with *Clal* and *XhoI*. This fragment was cloned into the *Clal-XhoI* digested pBluescript SK- (Stratagene, San Diego, CA) to create pBluescript/wob (- *NarI* fragment). This  
5 plasmid was digested with *EcoRI* and *NarI* to retrieve the wobbled *gag* sequence and the *EcoRI-NarI* fragment cloned into the *EcoRI-NarI* digested pCI/5' truncated *g/p* described in section C above in order to create pCI/5'truncated wob *g/p*.

E. Creation of a 5' and 3' truncated *gag/pol* construct in pCI and pSCV 10

10 This example describes the construction of the 5' and 3' truncated *gag/pol* construct in the pCI vector where the 5' truncation as described in section C is combined with the following 3'truncation upstream of the stop codon eliminating the DNA sequence coding for the last 28 amino acids of the *pol* protein.

Briefly, the plasmid pCI/5'truncated *g/p* described in section C was  
15 linearized with the restriction enzyme *SmaI* which is located 84 bases upstream of the *gag/pol* termination codon in the open reading frame of *gag/pol*. The linearized plasmid was ligated to the oligonucleotide 5' TAAGCGGCCG CTTA (SEQ ID NO: 45). This oligonucleotide is self-complementary and forms a palindromic duplex containing a TAA termination codon and a *NotI* restriction endonuclease site. After  
20 ligation of 100ng vector and 5μM oligo in the presence of T4 DNA ligase, the reaction was purified by GeneClean and digested with *SmaI* to recut any vector that did not contain an insert. The reaction was used to transform XL1 Blue *E. coli* (Stratagene, San Diego, CA) and plasmid DNA from a correct clone was then digested with *NotI*. *NotI* cuts in the inserted oligonucleotide as well as just upstream of the SV40  
25 termination/polyadenylation site of the pCI vector. The digested plasmids were purified by GeneClean and religated to recircularize. Bacteria were transformed and clones were identified which deleted the sequences between the *NotI* site introduced by the oligonucleotide and the *NotI* site in the pCI vector. These sequences include sequences encoding the last 28 amino acids of *gag/pol* as well as MoMLV sequences and vector  
30 sequences carried over from pSCV10. The resulting *gag/pol* construct was named pCI-GPM. The identically shortened *gag/pol* region was cloned by standard techniques into a pSCV10 background expression cassette. This expression plasmid was named pSCV10/5',3'tr.



F. Creation of a 5' and 3' truncated and wobbled *gag/pol* construct in pCI

This example describes the construction of the 5' and 3' truncated and wobbled *gag/pol* construct in the pCI vector combining the 5' truncation and wobbled *gag/pol* sequence from section D above with the 3' truncation described in section E  
5 above.

Briefly, pCI/5'truncated wob g/p was linearized with *SmaI* and all further steps leading to the 3' truncation of *gag/pol* were carried out as described in section E above, leading to the 5' and 3' truncated and wobbled *gag/pol* construct in pCI named pCI-WGPM.

10

EXAMPLE 14

CONSTRUCTION AND TESTING TITER POTENTIAL OF PCLs WITH VARIOUS  
COMBINATIONS OF *GAG/POL* AND *ENV* EXPRESSION CASSETTES RESULTING IN PCLs  
15 WITH VARIOUS DEGREES OF DNA SEQUENCE OVERLAP BETWEEN THE RETROVIRAL  
COMPONENTS: *GAG/POL*, *ENV* AND RETROVIRAL VECTOR

This example describes the production of PCLs based on various combinations of the *gag/pol* and *env* expression cassettes described above and in  
20 Examples 12 and 13. The three unmodified retroviral components *gag/pol*, *env* and retroviral vector (PCT Application No. WO 92/05266) result in three areas of sequence overlap (area 1-3) in a VCL as shown in Figure 22A. PCLs/VCLs with reduced sequence overlap can be produced with elimination of any combination of these three sequence overlap areas for example, a PCL/VCL may eliminate sequence overlap of  
25 area 1, area 2 or area 3 only, a combination of any two or all three areas. Production and potential titer analysis of PCLs with all three overlaps eliminated (Figure 22 C) as well as PCLs with the first area of overlap reduced and area 2 and 3 eliminated (Figure 22 B) are described below. A critical issue in the production of PCLs with reduced sequence overlap is the maintenance of high titer potential. Therefore, the titer potential  
30 of the PCLs with reduced sequence overlap were analyzed and compared extensively to existing PCLs with unmodified PCL components such as the DA and HA PCLs described in PCT Application No. WO 92/05266.

35 A. Production of PCLs with one area of sequence overlap between PCL components

This example describes the production of PCLs with the *gag/pol* expression plasmid cassette pSCV10/5',3'tr. or pCI-GPM described in Example 13 E

and the *env* expression plasmid pCMV-b/envam described in Example 12 E. PCLs with these *gag/pol* and *env* expression plasmids in conjunction with a retroviral vector derived from pBA-5b (Example 9) result in VCLs where sequence overlap areas 2 and 3 are eliminated and area 1 is reduced (Figure 22 B). The cell lines HT 1080 (ATCC #CCL 121) and D17 (ATCC #CCL 183) were used as parent cell lines to establish the PCLs.

Briefly, either *gag/pol* plasmid pSCV10/5',3'tr. or pCI-GMP was co-transfected together with a phleomycin<sup>r</sup> expressing marker plasmid into HT 1080 and D17 cells, respectively, via CaPO<sub>4</sub>-precipitation using the ProFection kit from Promega according to manufacturer's protocol (Promega, Madison, WI). The transfected cells were selected with media containing zeocin<sup>TM</sup> (Invitrogen, Carlsbad, CA) at a concentration of 150 mg/ml for HT 1080 cells and 170 mg/ml for D17 cells until untransfected control cells were dead. HT 1080 and D17 *gag/pol* pools were dilution cloned into 96-well microtiter plates according to standard protocols where clonality was ensured by seeding cell densities that yield a maximum of 30% of wells with cell growth. HT 1080 and D17 derived *gag/pol* intermediate clones were isolated and analyzed for intracellular p30 expression levels in a standard Western blot using primary p30-specific goat antibodies and secondary, HRP-labeled rabbit anti-goat antibodies. These *gag/pol* clones were compared to HTSCV21 and D17 4-15 which are the IIT 1080 and D17 *gag/pol* intermediate clones for the PCLs HA and DA, respectively (PCT #WO 92/05266). DA and HA have all three areas of sequence overlap (Figure 22 A). Results of the p30 Western are shown below in Table 6.

**Table 6:**

HT 1080 and D17 derived clones screened for intracellular p30 levels after introduction of *gag/pol* expression cassettes pSCV10/5',3'tr. or pCI-GPM

Gag/pol intermediates	#clones screened for p30	#clones positive for p30 - (%)	p30 expression levels
D17gag/pol (pCI-GPM)	80	32 (40%)	3-4 clones have p30 levels comparable to D17 4-15
HT 1080gag/pol (pSCV10/5',3'tr.)	100	24 (24%)	15 clones have p30 levels comparable or higher than HTSCV21

The 18 HT 1080 and 12 D17 gag/pol intermediates with the highest p30 expression levels were analyzed for titer potential.

Briefly, a retroviral ecotropic *env* expressing vector and a retroviral vector coding for  $\beta$ -gal and neo<sup>r</sup> were co-introduced into the gag/pol intermediate clones, transient and stable supernatants harvested and  $\beta$ -gal titers determined on 3T3 target cells using either a standard blue X-gal staining procedure or a "Galactolight assay." Briefly, this assay allows rapid determination of  $\beta$ -gal vector titers by chemiluminescent detection of transfer of  $\beta$ -gal expression to HT 1080 target cells. (Tripix, New Bedford, MA). Expression was compared to a standard curve for transfer of expression versus titer generated by serial dilutions of a known titer reference  $\beta$ -gal vector.

The titer results for the HT 1080 gag/pol intermediates are shown below in Table 7.

15

**Table 7:**  
Transient  $\beta$ -gal titers from transduced pools of HT 1080 gag/pol intermediates

Clone#	Transient $\beta$ -gal titer (CFU/ml)	x-fold titer decrease (HTSCV21:HT 1080 gag/pol intermediate)
1	178	11
2	750	3
6	345	6
12	728	3
14	545	4
18	113	18
38	263	8
41	1100	2
42	(3)	(660)
47	83	24
53	573	3
57	95	21
59	850	2
62	518	4
67	440	5
69	0	-
70	375	5
90	1300	2
HTSCV21 1975		

11 out of 18 clones gave a titer potential that was 2-6 fold lower in comparison to HTSCV21. The titer results for the D17 gag/pol intermediate clones are shown below in Table 8.

20

5

**Table 8:**

Transient  $\beta$ -gal titers from transduced pools of D17gag/pol intermediates and stable  $\beta$ -gal titers from transduced and G-418 selected D17gag/pol intermediates

Clone#	Transient $\beta$ -gal titer (CFU/ml)	x-fold titer decrease (D17 4-15: D17 gag/pol intermediates)	Stable $\beta$ -gal titer (light units)	x-fold titer decrease
2	165	473	80.1	14
10	95	821	44.3	25
42	270	289	27.8	40
55	990	79	246.7	5
60	220	354	67.8	16
65	495	158	280.5	4
71	605	129	77.0	14
72	0	-	95.9	12
74	1.7E4	5	1497.3	no decrease
75	2100	37	1180.3	no decrease
84	3400	23	300.3	4
92	1600	49	2013.7	no decrease
D17 4-15	7.8E4	1112.3		

The transient titers show a strong decrease in titer potential when compared to D17 4-15, but for the stable titers, six out of the 12 gag/pol intermediates show 0-6 fold decrease in comparison to D17 4-15.

10 A total of 6 D17 and 4 HT 1080 gag/pol intermediates with the highest titer potential were co-transfected with the *env* expression plasmid pCMV-b/envam described in Example 12 and a methotrexate<sup>r</sup> expressing marker plasmid into the HT 1080 and D17gag/pol intermediate clones via CaPO<sub>4</sub>-precipitation using the ProFection kit from Promega according to manufacturer's protocol (Promega, Madison, WI). The  
15 transfected cells were selected with media containing methotrexate at a concentration of  $2 \times 10^{-7}$  M until untransfected control cells were dead. HT 1080 and D17 derived PCL pools were dilution cloned into 96-well microtiter plates according to standard protocols where clonality was ensured by seeding cell densities that yield a maximum of 30% of wells with cell growth. Several hundred HT 1080 and D17 derived PCL clones named  
20 HAI1 and DAI1, respectively, were isolated and analyzed for titer potential.

Briefly, five rounds of titer potential analysis were carried out using various retroviral vectors. The DA or HA PCL controls (PCT #WO 92/05266) were included as a reference for high titer potential PCLs. In the first round, the PCL clones were transduced in 24-well plates with the  $\beta$ -gal coding retroviral vector DX/ND7 (WO

95/16852) at an moi of 5-10 in the presence of 8 µg/ml polybrene, transient supernatants harvested, filtered (0.45 µm), HT 1080 target cells transduced and transient β-gal titer determined using a standard Galactolight procedure following manufacturer's instructions (Tropix, Bedford, MA). In the second round, the same transduction assay as described for the first round was repeated with the top clones from round one using standardized PCL cell numbers. In the following titer potential analysis rounds, the top clones from round two were used to transduce with several retroviral vectors, supernatant from transient and stable pools were harvested, filtered, HT 1080 target cells transduced, and transient and stable titers determined.

Data on the titer potential analysis of the second round of screening is shown below in Table 9 on a small selection of representative DAII and HAII PCL clones.

**Table 9:**

Transient β-gal titer on VCL pools from transduced HAII and DAII PCLs determined by Galactolight readout.

Clone#	Transient β-gal titer (Galactolight, light units)	x-fold decrease in titer potential (DA:DAII, HA:HAII)
<b>D-17 based PCLs called DAII</b>		
DAII (based on pCI-GPM#74 intermediate):		
20	3	-
30	69	14
47	19	51
49	1	-
55	145	7
60	1	-
67	8	-
70	45	22
DA	978	
DAII (based on pCI-GPM#75 intermediate):		
7	47	19
32	202	5
40	27	33
60	15	61
70	7	-
72	1	-
DA	901	
<b>HT-1080 based PCLs called HAII</b>		
HAII (based on pSCV10/5'3'tr.intermediate #12):		
6	147	10
11	8	-

12	56	27
18	45	34
44	113	13
51	2	-
54	2	-
56	83	18
57	115	13
65	133	11
66	104	15
78	195	8
86	125	12
87	77	20
88	259	6
90	196	8
91	91	17
HA	1508	
HAII(based on pSCV10/5'3'tr.#41):		
4	48	31
9	84	18
15	157	10
18	174	9
37	111	14
55	357	4
58	140	11
75	164	9
92	57	28
HA	1570	

The top DAII and HAII PCL clones gave a 4-5 fold reduced titer potential when measured as a transient  $\beta$ -gal pool. A large percentage of these DAII and HAII PCL clones gave a 10-15 fold decrease in titer potential.

The top DAII and HAII clones were further tested for their titer potential using various retroviral vectors. Table 10 below shows a summary of titer potentials on VCL pools of the top HAII clones, with HAII#41#55 as the overall best PCL.

Table 10:

PCL clone#	transient factor VIII	stable PLAP (BAAP)	stable neo (BAAP)	stable neo (KT-1)	transient hGH	transient factor VIII
HAI:						
12#78	1.1E5	5.0E4	1.6E4	5.3E5	36,000	7.7E4
12#86	8.8E4	6.6E4	1.1E4	8.4E5	29,200	7.1E4
12#88	1.1E4	3.2E4	8.6E4	8.2E5	34,500	1.1E5
12#90	9.4E4	1.0E5	6.4E4	9.4E5	37,700	1.1E5
41#18	7.0E4	6.2E4	2.0E4	1.3E5	34,400	1.8E5
41#55	9.9E4	6.3E5	1.7E5	1.1E6	44,000	1.3E5
41#58	8.9E4	4.8E4	8.8E3	5.1E5	38,700	4.3E4
41#75	1.1E5	1.4E5	3.2E4	8.7E5	34,300	1.5E5
HA	5.3E5 1.3E5	4.1E4	2.1E4 1.6E4	5.0E5 1.1E7	38,300 39,300	3.7E5
DA	3.9E4 7.3E3	1.0E6	4.0E5 8.4E6	5.9E5 4.8E6	45,700 52,500	1.9E5

Values in italics must be compared to the control PCL values (DA, HA) in italics

5 Differences in titer potential were observed, depending not only on which PCL clone was used but also which gene of interest was expressed in the retroviral vector.

10 Comparison of the 8 top HAI clones with titers on VCL pools from various rounds of titer potential assays. The B-domain deleted factor VIII, human placental alkaline phosphatase plus neo' (BAAP), human growth hormone (hGH) and HIVenv/rev plus neo' (KT-1) expressing retroviral vectors were used to transduce the HAI PCLs. Transient and stable supernatants were tested on HT 1080 target cells. The readout for hGH is in units and the other titers are in CFU/ml.

15 B. Production of PCLs without any sequence overlap between PCL components

This example describes the production of PCLs with the *gag/pol* expression plasmid cassette pCI-WGPM described in Example 13 F and the *env* expression plasmid pCMV-b/envam described in Example 12 E. PCLs with these *gag/pol* and *env* expression plasmids in conjunction with the retroviral vector derived from pBA-5b (Example 9) result in producer cell lines where sequence overlap between all three areas of homology is completely eliminated (Figure 22 C). The cell lines HT 1080 (ATCC #CCL 121) and D17 (ATCC #CCL 183) were used as parent cell lines to establish the PCLs.

- Briefly, *gag/pol* plasmid pCI-WGPM was co-transfected together with a phleomycin<sup>r</sup> expressing marker plasmid into HT 1080 and D17 cells, selected and dilution cloned as described above. HT 1080 and D17 derived clones were isolated and analyzed for intracellular p30 expression levels as described above. Results of the p30 Western are shown below in Table 11.

Table 11:

- 10 HT 1080 and D17 derived clones screened for intracellular p30 levels after introduction of *gag/pol* expression cassette pCI-WGPM

Gag/pol intermediates	#clones screened for p30	#clones positive for p30 - (%)	p30 expression levels
D-17g/p (pCI-WGPM)	82	36 (44%)	3-4 clones have p30 levels comparable to D17 4-15
HT-1080g/p (pCI-WGPM)	96	26 (27%)	3-4 clones have p30 levels that are comparable to HTSCV21

- 15 The 12 HT 1080 and 22 D17 *gag/pol* intermediates with the highest p30 expression levels were analyzed for titer potential as described above. The titer results for the HT 1080 *gag/pol* intermediates are shown below in Table 12.

Table 12:

- 20 Transient  $\beta$ -gal titers from transduced pools of HT 1080 *gag/pol* intermediates (pCI-WGPM)



Clone#	Transient $\beta$ -gal titer (CFU/ml)	x-fold titer decrease (HTSCV21:HT 1080 gag/pol intermediate)
10	217	>9
12	28	>71
23	670	>3
29	565	>4
34	950	>2
35	398	>5
45	280	>7
52	670	>3
53	600	>3
71	590	>3
86	480	>4
87	55	>36
HTSCV21	>2000	

The Galactolight readout for HTSCV10 was out of the range with >2000, therefore the above shown decrease in titer potential is likely to be higher. The titer results for the D17 gag/pol intermediates are shown below in Table 13.

5

Table 13:

Transient  $\beta$ -gal titers from transduced pools of D17gag/pol (D17 g/p) intermediates and stable  $\beta$ -gal titers from transduced and G-418 selected pools of D17gag/pol intermediates

10

Clone#	Transient $\beta$ -gal titer (CFU/ml)	x-fold decrease (D17 4- 15:D17g/ p inter.)	Stable $\beta$ - gal titer (CFU/ml)	x-fold decrease (D17 4-15: D17g/p inter.)	Transient $\beta$ -gal (CFU/ml)	x-fold decrease (D17 4- 15:D17g/p inter.)
1	0	-				
3	40	>100				
6	20	>200				
14	10	>400				
21	10	>400				
22	1380	>3	800	>5	2.1E4	9
27	30	>133				
41	100	>40				
47	30	>133	730	>6	90	2111
48	30	>133				
49	500	>8	680	>6	9.4E3	20
50	140	>29				
51	10	>400				
56	600	>7	320	>13	1.8E3	105
57	230	>17			1.3E3	146
60	380	>11	580	>7	1.0E4	190
65	0	-				
66	470	>9	330	>12	1.1E3	172
70	30	>133				
73	320	>13	1.05E4	0		

76	40	>100		
79	20	>200		
D17 4-15	>4000*	>4000*		1.9E5

\*=out of range

The titer potential measured within the range indicates decreases in titer potential of 10-200 fold in most clones.

A total of 6 D17 and 4 HT 1080 gag/pol intermediates with the highest titer potential were co-transfected with the *env* expression plasmid pCMV-b/envam, pools selected and dilution cloned as described above. Several hundred HT 1080 and D17 derived PCL clones named HAI1wob and DAI1wob, respectively, were isolated and analyzed for titer potential.

Briefly, several rounds of titer potential analysis were carried out using various retroviral vectors. The DA or HA PCL (PCT #WO 92/05266) controls were included as a reference for high titer potential PCLs. In the first round, the PCL clones were transduced in 24-well plates with the  $\beta$ -gal coding retroviral vector DX/ND7 (WO 95/16852) at an moi of 5-10 in the presence of 8  $\mu$ g/ml polybrene, transient supernatants harvested, filtered (0.45  $\mu$ m), HT 1080 target cells transduced and transient  $\beta$ -gal titer determined using a standard Galactolight transfer of expression procedure described previously. In the second round, the same transduction assay as described for the first round was repeated with the top clones from round one using standardized PCL cell numbers. In the third round, the top clones from round two were used to transduce with several retroviral vectors, supernatant from transient and stable pools harvested, filtered, HT 1080 target cells transduced and titers determined.

Data on the titer potential analysis of the first and second round of screening is shown below in Table 14 on a small selection of representative DAI1 and HAI1 PCL clones.

25

**Table 14:**

Transient  $\beta$ -gal titer on VCL pools from transduced HAI1 and DAI1 PCLs determined by Galactolight readout.

Clone#	Transient $\beta$ -gal titer (Galactolight, light units)	x-fold decrease in titer potential (DA:DAI1wob or HA:HAI1wob)
--------	---	---

**D-17 based PCLs called DAI1wob**

DAI1wob (pCI-WGPM#60):

7	21	27
11	6	93
21	2	279

30	14	40
33	51	11
41	30	19

DA 558

DAIIwob (pCI-WGPM#22):

5	148	0
8	28	5
28	14	11
56	15	10
78	39	4
97	10	15

DA 153

#### HT-1080 based PCLs called HAIWob

HAIWob (pCI-WGPM)#34:

4	8	128
7	9	114
35	7	147
43	4	257
53	5	205
65	9	114
66	10	103
77	19	54
79	6	171
80	4	257
95	4	257
105	2	500
115	9	114
118	6	171

HA 1026

5 The best DAIIwob PCL clone shows a 4-fold reduction in titer but most clones show >10-fold reduction. The best HAIWob PCL clone shows a 50-fold reduced titer potential and most HAIWob clones have >100-fold reduced titer potential. In general, the DAII wob and HAIWob PCL clones gave in average about a 5-50 fold lower titer potential when compared to DAII and HAI PCLs.

10 From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

## SEQUENCE LISTING

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  - (D) SOFTWARE: PatentIn Release #1.0. Version #1.25
- (vi) CURRENT APPLICATION DATA:
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  - (B) FILING DATE: 06-MAY-1997
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 8332 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCCAGTCC TCCGATTGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AACCTCTTG	60
CAGTTGCATC CGACTTGTGG TCTCGCTGTT CCTTGGGAGG GTCTCCTCTG AGTGATTGAC	120
TACCCGTCAG CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCGGGAGA CCCCTGCCCA	180
GGGACCACCG ACCCACCACC GGGAGGTAAG CTGGCCAGCA ACTTATCTGT GTCTGTCCGA	240
TTGTCTAGTG TCTATGACTG ATTTTATGCG CCTGCGTCGG TACTAGTTAG CTAAGTAGCT	300
CTGTATCTGG CGGACCCGTG GTGGAAGTGA CGAGTTCGGA ACACCCGGCC GCAACCCTGG	360
GAGACGTCCC AGGGACTTCG GGGGCCGTTT TTGTGGCCCG ACCTGAGTCC AAAAATCCCG	420
ATCGTTTTGG ACTCTTTGGT GCACCCCCCT TAGAGGAGGG ATATGTGGTT CTGGTAGGAG	480
ACGAGAACCT AAAACAGTTC CCGCCTCCGT CTGAATTTTT GCTTTCGGTT TGGGACCGAA	540
GCCGCGCCGC GCGTCTTGTC TGCTGCAGCA TCGTTCTGTG TTGTCTCTGT CTGACTGTGT	600
TTCTGTATTT GTCTGAGAAT ATGGGCCAGA CTGTTACCAC TCCCTTAAGT TTGACCTTAG	660
GTCAGTGAA AGATGTCGAG CGGATCGCTC ACAACCAGTC GGTAGATGTC AAGAAGAGAC	720
GTTGGGTTAC CTTCTGCTCT GCAGAATGGC CAACCTTTAA CGTCGGATGG CCGCGAGACG	780
GCACCTTTAA CCGAGACCTC ATCACCAGG TTAAGATCAA GGTCTTTTCA CCTGGCCCGC	840
ATGGACACCC AGACCAGGTC CCCTACATCG TGACCTGGGA AGCCTTGGCT TTTGACCCCC	900
CTCCCTGGGT CAAGCCCTTT GTACACCCTA AGCCTCCGCC TCCTCTTCCT CCATCCGCCC	960
CGTCTCTCCC CTTGAACCT CCTCGTTCGA CCCCGCCTCG ATCCTCCCTT TATCCAGCCC	1020
TCACTCCTTC TCTAGGCGCC AAACCTAAAC CTCAAGTTCT TTCTGACAGT GGGGGGCCGC	1080
TCATCGACCT ACTTACAGAA GACCCCCCGC CTTATAGGGA CCCAAGACCA CCCCCTTCG	1140
ACAGGGACGG AAATGGTGGA GAAGCGACCC CTGCGGGAGA GGCACCGGAC CCCTCCCCAA	1200
TGGCATCTCG CCTACGTGGG AGACGGGAGC CCCCTGTGGC CGACTCCACT ACCTCGCAGG	1260
CATTCCCCCT CCGCGCAGGA GGAAACGGAC AGCTTCAATA CTGGCCGTTT TCCTCTTCTG	1320
ACCTTTACAA CTGGAAAAAT AATAACCCTT CTTTTTCTGA AGATCCAGGT AACTGACAG	1380
CTCTGATCGA GTCTGTTCTC ATCACCATC AGCCACCTG GGACGACTGT CAGCAGCTGT	1440
TGGGGACTCT GCTGACCGGA GAAGAAAAAC AACGGGTGCT CTTAGAGGCT AGAAAGGCGG	1500

TGCGGGGCGA TGATGGGCGC CCCACTCAAC TGCCCAATGA AGTCGATGCC GCTTTTCCCC	1560
TCGAGCGCCC AGACTGGGAT TACACCACCC AGGCAGGTAG GAACCACCTA GTCCACTATC	1620
GCCAGTTGCT CCTAGCGGGT CTCCAAAACG CGGGCAGAAG CCCCACCAAT TTGGCCAAGG	1680
TAAAAGGAAT AACACAAGGG CCCAATGAGT CTCCTCGGC CTTCCTAGAG AGACTTAAGG	1740
AAGCCTATCG CAGGTACACT CCTTATGACC CTGAGGACCC AGGGCAAGAA ACTAATGTGT	1800
CTATGTCTTT CATTTGGCAG TCTGCCCCAG ACATTGGGAG AAAGTTAGAG AGGTTAGAAG	1860
ATTAAAAAA CAAGACGCTT GGAGATTTGG TTAGAGAGGC AGAAAAGATC TTTAATAAAC	1920
GAGAAACCCC GGAAGAAAGA GAGGAACGTA TCAGGAGAGA AACAGAGGAA AAAGAAGAAC	1980
GCCGTAGGAC AGAGGATGAG CAGAAAGAGA AAGAAAGAGA TCGTAGGAGA CATAGAGAGA	2040
TGAGCAAGCT ATTGGCCACT GTCGTTAGTG GACAGAAACA GGATAGACAG GGAGGAGAAC	2100
GAAGGAGGTC CCAACTCGAT CGCGACCAGT GTGCCTACTG CAAAGAAAAG GGGCACTGGG	2160
CTAAAGATTG TCCCAAGAAA CCACGAGGAC CTCGGGGACC AAGACCCAG ACCTCCCTCC	2220
TGACCCTAGA TGACTIONGGA GGTGAGGGTC AGGAGCCCCC CCCTGAACCC AGGATAACCC	2280
TCAAAGTCGG GGGGCAACCC GTCACCTTCC TGGTAGATAC TGGGGCCCAA CACTCCGTGC	2340
TGACCCAAAA TCCTGGACCC CTAAGTGATA AGTCTGCCTG GGTCCAAGGG GCTACTGGAG	2400
GAAAGCGGTA TCGCTGGACC ACGGATCGCA AAGTACATCT AGCTACCGGT AAGGTCACCC	2460
ACTCTTTCCT CCATGTACCA GACTGTCCCT ATCCTCTGTT AGGAAGAGAT TTGCTGACTA	2520
AACTAAAAGC CCAAATCCAC TTTGAGGGAT CAGGAGCTCA GGTTATGGGA CCAATGGGGC	2580
AGCCCCTGCA AGTGTGACC CTAAATATAG AAGATGAGCA TCGGCTACAT GAGACCTCAA	2640
AAGAGCCAGA TGTTTCTCTA GGGTCCACAT GGCTGTCTGA TTTTCCTCAG GCCTGGGCGG	2700
AAACCGGGGG CATGGGACTG GCAGTTCGCC AAGCTCCTCT GATCATACCT CTGAAAGCAA	2760
CCTCTACCCC CGTGTCCATA AAACAATACC CCATGTCACA AGAAGCCAGA CTGGGGATCA	2820
AGCCCCACAT ACAGAGACTG TTGGACCAGG GAATACTGGT ACCCTGCCAG TCCCCCTGGA	2880
ACACGCCCTT GCTACCCGTT AAGAAACCAG GGAATAATGA TTATAGGCCT GTCCAGGATC	2940
TGAGAGAAGT CAACAAGCGG GTGGAAGACA TCCACCCAC CGTGCCCAAC CCTTACAACC	3000
TCTTGAGCGG GCTCCCACCG TCCCACCAGT GGTACACTGT GCTTGATTTA AAGGATGCCT	3060
TTTTCTGCCT GAGACTCCAC CCCACCAGTC AGCCTCTCTT CGCCTTTGAG TGGAGAGATC	3120

CAGAGATGGG AATCTCAGGA CAATTGACCT GGACCAGACT CCCACAGGGT TTCAAAAACA	3180
GTCCCACCCT GTTTGATGAG GCACTGCACA GAGACCTAGC AGACTTCCGG ATCCAGCACC	3240
CAGACTTGAT CCTGCTACAG TACGTGGATG ACTTACTGCT GGCCGCCACT TCTGAGCTAG	3300
ACTGCCAACA AGGTACTCGG GCCCTGTTAC AAACCCTAGG GAACCTCGGG TATCGGGCCT	3360
CGGCCAAGAA AGCCCAAATT TGCCAGAAAC AGGTCAAGTA TCTGGGGTAT CTTCTAAAAG	3420
AGGGTCAGAG ATGGCTGACT GAGGCCAGAA AAGAGACTGT GATGGGGCAG CCTACTCCGA	3480
AGACCCCTCG ACAACTAAGG GAGTTCCTAG GGACGGCAGG CTTCTGTGCG CTCTGGATCC	3540
CTGGGTTTGC AGAAATGGCA GCCCCCTTGT ACCCTCTCAC CAAAACGGGG ACTCTGTTTA	3600
ATTGGGGCCC AGACCAACAA AAGGCCTATC AAGAAATCAA GCAAGCTCTT CTAAGTCCC	3660
CAGCCCTGGG GTTGCCAGAT TTGACTAAGC CCTTTGAACT CTTTGTGAC GAGAAGCAGG	3720
GCTACGCCAA AGGTGTCTTA ACGCAAAAC TGGGACCTTG GCGTCGGCCG GTGGCCTACC	3780
TGTCCAAAAA GCTAGACCCA GTAGCAGCTG GGTGGCCCCC TTGCCTACGG ATGGTAGCAG	3840
CCATTGCCGT ACTGACAAAG GATGCAGGCA AGCTAACCAT GGGACAGCCA CTAGTCATTC	3900
TGGCCCCCCA TGCAGTAGAG GCACTAGTCA AACAACCCCC CGACCGCTGG CTTTCCAACG	3960
CCCGGATGAC TCACTATCAG GCCTTGCTTT TGGACACGGA CCGGGTCCAG TTCGGACCGG	4020
TGGTAGCCCT GAACCCGGCT ACGCTGCTCC CACTGCCTGA GGAAGGGCTG CAACACAAC	4080
GCCTTGATAT CCTGGCCGAA GCCCACGGAA CCCGACCGA CCTAACGGAC CAGCCGCTCC	4140
CAGACGCCGA CCACACCTGG TACACGGATG GAAGCAGTCT CTTACAAGAG GGACAGCGTA	4200
AGGCGGGAGC TGCGGTGACC ACCGAGACCG AGGTAATCTG GGCTAAAGCC CTGCCAGCCG	4260
GGACATCCGC TCAGCGGGCT GAAGTGATAG CACTCACCCA GGCCCTAAAG ATGGCAGAAG	4320
GTAAGAAGCT AAATGTTTAT ACTGATAGCC GTTATGCTTT TGCTACTGCC CATATCCATG	4380
GAGAAATATA CAGAAGGCGT GGGTTGCTCA CATCAGAAGG CAAAGAGATC AAAAATAAAG	4440
ACGAGATCTT GGCCCTACTA AAAGCCCTCT TTCTGCCCAA AAGACTTAGC ATAATCCATT	4500
GTCCAGGACA TCAAAAGGGA CACAGCGCCG AGGCTAGAGG CAACCGGATG GCTGACCAAG	4560
CGGCCCCGAA GGCAGCCATC ACAGAGACTC CAGACACCTC TACCCTCCTC ATAGAAAATT	4620
CATCACCCCTA CACCTCAGAA CATTTTCATT ACACAGTGAC TGATATAAAG GACCTAACCA	4680
AGTTGGGGGC CATTTATGAT AAAACAAAGA AGTATTGGGT CTACCAAGGA AAACCTGTGA	4740
TGCCTGACCA GTTTACTTTT GAATTATTAG ACTTTCTTCA TCAGCTGACT CACCTCAGCT	4800

TCTCAAAAAT GAAGGCTCTC CTAGAGAGAA GCCACAGTCC CTA CTACATG CTGAACCGGG 4860  
ATCGAACACT CAAAAATATC ACTGAGACCT GCAAAGCTTG TGCACAAGTC AACGCCAGCA 4920  
AGTCTGCCGT TAAACAGGGA ACTAGGGTCC GCGGGCATCG GCCCGGCACT CATTGGGAGA 4980  
TCGATTTTAC CGAGATAAAG CCCGGATTGT ATGGCTATAA ATATCTTCTA GTTTTTATAG 5040  
ATACCTTTTC TGGCTGGATA GAAGCCTTCC CAACCAAGAA AGAAACCGCC AAGGTCGTAA 5100  
CCAAGAAGCT ACTAGAGGAG ATCTTCCCCA GGTTCGGCAT GCCTCAGGTA TTGGGAAGCTG 5160  
ACAATGGGCC TGCCTTCGTC TCCAAGGTGA GTCAGACAGT GGCCGATCTG TTGGGGATTG 5220  
ATTGGAAATT ACATTGTGCA TACAGACCCC AAAGCTCAGG CCAGGTAGAA AGAATGAATA 5280  
GAACCATCAA GGAGACTTTA ACTAAATTAA CGCTTGCAAC TGGCTCTAGA GACTGGGTGC 5340  
TCCTACTCCC CTTAGCCCTG TACCGAGCCC GCAACACGCC GGGCCCCCAT GGCCTCACCC 5400  
CATATGAGAT CTTATATGGG GCACCCCCGC CCCTTGTAAC CTTCCCTGAC CCTGACATGA 5460  
CAAGAGTTAC TAACAGCCCC TCTCTCCAAG CTCACTTACA GGCTCTCTAC TTAGTCCAGC 5520  
ACGAAGTCTG GAGACCTCTG GCGGCAGCCT ACCAAGAACA ACTGGACCGA CCGGTGGTAC 5580  
CTCACCCCTA CCGAGTCGGC GACACAGTGT GGGTCCGCCG ACACCAGACT AAGAACCTAG 5640  
AACCTCGCTG GAAAGGACCT TACACAGTCC TGCTGACCAC CCCACCGCC CTCAAAGTAG 5700  
ACGGCATCGC AGCTTGGATA CACGCCGCC ACGTGAAGGC TGCCGACCCC GGGGGTGGAC 5760  
CATCCTCTAG ACTGACATGG CGCGTTCAAC GCTCTCAAAA CCCCTTAAAA ATAAGGTAA 5820  
CCCGCGAGGC CCCCTAATCC CCTTAATTCT TCTGATGCTC AGAGGGGTCA GTACTGCTTC 5880  
GCCCCGCTCC AGTCCTCATC AAGTCTATAA TATCACCTGG GAGGTAACCA ATGGAGATCG 5940  
GGAGACGGTA TGGGCAACTT CTGGCAACCA CCCTCTGTGG ACCTGGTGGC CTGACCTTAC 6000  
CCCAGATTTA TGTATGTTAG CCCACCATGG ACCATCTTAT TGGGGGCTAG AATATCAATC 6060  
CCCTTTTTCT TCTCCCCCGG GGCCCCCTTG TTGCTCAGGG GGCAGCAGCC CAGGCTGTTC 6120  
CAGAGACTGC GAAGAACCTT TAACCTCCCT CACCCCTCGG TGCAAACTG CCTGGAACAG 6180  
ACTCAAGCTA GACCAGACAA CTCATAAATC AAATGAGGGA TTTTATGTTT GCCCCGGGCC 6240  
CCACCGCCCC CGAGAATCCA AGTCATGTGG GGGTCCAGAC TCCTTCTACT GTGCCTATTG 6300  
GGGCTGTGAG ACAACCGGTA GAGCTTACTG GAAGCCCTCC TCATCATGGG ATTCATCAC 6360  
AGTAAACAAC AATCTCACCT CTGACCAGGC TGTCCAGGTA TGCAAAGATA ATAAGTGGTG 6420



CAACCCCTTA GTTATTCGGT TTACAGACGC CGGGAGACGG GTTACTTCCT GGACCACAGG	6480
ACATTACTGG GGCTTACGTT TGTATGTCTC CGGACAAGAT CCAGGGCTTA CATTTGGGAT	6540
CCGACTCAGA TACCAAAATC TAGGACCCCG CGTCCCAATA GGGCCAAACC CCGTTCTGGC	6600
AGACCAACAG CCACTCTCCA AGCCCAAACC TGTTAAGTCG CCTTCAGTCA CCAAACCACC	6660
CAGTGGGACT CCTCTCTCCC CTACCCAACT TCCACCGGCG GGAACGGAAA ATAGGCTGCT	6720
AAACTTAGTA GACGGAGCCT ACCAAGCCCT CAACCTCACC AGTCCTGACA AAACCCAAGA	6780
GTGCTGGTTG TGTCTAGTAG CGGGACCCCC CTACTACGAA GGGGTTGCCG TCCTGGGTAC	6840
CTACTCCAAC CATACTCTG CTCCAGCCAA CTGCTCCGTG GCCTCCCAAC ACAAGTTGAC	6900
CCTGTCCGAA GTGACCGGAC AGGGACTCTG CATAGGAGCA GTTCCCAAAA CACATCAGGC	6960
CCTATGTAAT ACCACCCAGA CAAGCAGTCG AGGGTCCTAT TATCTAGTTG CCCCTACAGG	7020
TACCATGTGG GCTTGTAGTA CCGGGCTTAC TCCATGCATC TCCACCACCA TACTGAACCT	7080
TACCACTGAT TATTGTGTTT TTGTCGAACT CTGGCCAAGA GTCACCTATC ATTCCCCCAG	7140
CTATGTTTAC GGCCTGTTTG AGAGATCCAA CCGACACAAA AGAGAACCGG TGTCGTTAAC	7200
CCTGGCCCTA TTATTGGGTG GACTAACCAT GGGGGGAATT GCCGCTGGAA TAGGAACAGG	7260
GACTACTGCT CTAATGGCCA CTCAGCAATT CCAGCAGCTC CAAGCCGCAG TACAGGATGA	7320
TCTCAGGGAG GTTGAAAAAT CAATCTCTAA CCTAGAAAAG TCTCTCACTT CCCTGTCTGA	7380
AGTTGTCCTA CAGAATCGAA GGGGCCTAGA CTTGTTATTT CTAAAAGAAG GAGGGCTGTG	7440
TGCTGCTCTA AAAGAAGAAT GTTGCTTCTA TGCGGACCAC ACAGGACTAG TGAGAGACAG	7500
CATGGCCAAA TTGAGAGAGA GGCTTAATCA GAGACAGAAA CTGTTTGAGT CAACTCAAGG	7560
ATGGTTTGAG GGAAGTTTAA ACAGATCCCC TTGGTTTACC ACCTTGATAT CTACCATTAT	7620
GGGACCCCTC ATTGTACTCC TAATGATTTT GCTCTTCGGA CCCTGCATTC TTAATCGATT	7680
AGTCCAATTT GTTAAAGACA GGATATCAGT GGTCCAGGCT CTAGTTTTGA CTCAACAATA	7740
TCACCAGCTG AAGCCTATAG AGTACGAGCC ATAGATAAAA TAAAGATTT TATTTAGTCT	7800
CCAGAAAAAG GGGGGAATGA AAGACCCAC CTGTAGGTTT GGCAAGCTAG CTTAAGTAAC	7860
GCCATTTTGC AAGGCATGGA AAAATACATA ACTGAGAATA GAGAAGTTCA GATCAAGGTC	7920
AGGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG	7980
CCCCGGCTCA GGGCCAAGAA CAGATGGAAC AGCTGAATAT GGGCCAAACA GGATATCTGT	8040
GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC AAGAACAGAT GGTCCCCAGA TGCGGTCCAG	8100

CCCTCAGCAG TTTCTAGAGA ACCATCAGAT GTTTCAGGG TGCCCAAGG ACCTGAAATG 8160  
ACCCTGTGCC TTATTTGAAC TAACCAATCA GTTCGCTTCT CGCTTCTGTT CGCGCGCTTC 8220  
TGCTCCCGA GCTCAATAAA AGAGCCCACA ACCCCTCACT CGGGGCGCCA GTCCTCCGAT 8280  
TGACTGAGTC GCCCGGGTAC CCGTGTATCC AATAAACCT CTTGCAGTTG CA 8332

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTAACAGTC TGGCCGAAT TCTCAGACAA ATACAG 36

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGTATTTGT CTGAGAATTA AGGCTAGACT GTTACCAC 38

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATATATATAT ATCGATACCA TG 22

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCGCCAAAC CTAAAC

16

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Lys Asn Tyr Pro

5

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCATCCTCT GGACGGACAT G

21

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCCGGCCGT GGACGGACAT G

21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 449 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 20..442

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATATATATAT ATCGATACC ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC CTC	52
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu	
1 5 10	
ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC	100
Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser	
15 20 25	
GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG	148
Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp	
30 35 40	
CCC ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT	196
Pro Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp	
45 50 55	
CTG ATT ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC	244
Leu Ile Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly	
60 65 70 75	
CAT CCC GAT CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC	292
His Pro Asp Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe	
80 85 90	
GAT CCA CCA CCT TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA	340
Asp Pro Pro Pro Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro	
95 100 105	
CCC CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC	388

Pro Leu Pro Pro Ser Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser  
 110 115 120

ACA CCA CCC AGG AGC AGC TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC 436  
 Thr Pro Pro Arg Ser Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly  
 125 130 135

GCC AAA CCTAAAC 449  
 Ala Lys  
 140

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 141 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp  
 1 5 10 15

Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys  
 20 25 30

Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val  
 35 40 45

Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val  
 50 55 60

Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val  
 65 70 75 80

Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp  
 85 90 95

Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser  
 100 105 110

Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser  
 115 120 125

Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys  
 130 135 140

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 420 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 1..420

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG GGC CAG ACT GTT ACC ACT CCC TTA AGT TTG ACC TTA GGT CAC TGG	48
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp	
1 5 10 15	
AAA GAT GTC GAG CGG ATC GCT CAC AAC CAG TCG GTA GAT GTC AAG AAG	96
Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys	
20 25 30	
AGA CGT TGG GTT ACC TTC TGC TCT GCA GAA TGG CCA ACC TTT AAC GTC	144
Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val	
35 40 45	
GGA TGG CCG CGA GAC GGC ACC TTT AAC CGA GAC CTC ATC ACC CAG GTT	192
Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val	
50 55 60	
AAG ATC AAG GTC TTT TCA CCT GGC CCG CAT GGA CAC CCA GAC CAG GTC	240
Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val	
65 70 75 80	
CCC TAC ATC GTG ACC TGG GAA GCC TTG GCT TTT GAC CCC CCT CCC TGG	288
Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp	
85 90 95	
GTC AAG CCC TTT GTA CAC CCT AAG CCT CCG CCT CCT CTT CCT CCA TCC	336
Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser	
100 105 110	
GCC CCG TCT CTC CCC CTT GAA CCT CCT CGT TCG ACC CCG CCT CGA TCC	384
Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser	
115 120 125	
TCC CTT TAT CCA GCC CTC ACT CCT TCT CTA GGC GCC	420
Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala	
130 135 140	

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 140 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1           5           10           15
Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys
          20           25           30
Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
      35           40           45
Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
 50           55           60
Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
 65           70           75           80
Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
      85           90           95
Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
      100           105           110
Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
      115           120           125
Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala
      130           135           140

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(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2001 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

GGCCGACACC CAGAGTGGAC CATCCTCTGG ACGGACATGG CGCGTTCAAC GCTCTCAAAA      60
CCCCCTCAAG ATAAGATTAA CCCGTGGAAG CCCTTAATAG TCATGGGAGT CCTGTTAGGA      120
GTAGGGATGG CAGAGAGCCC CCATCAGGTC TTTAATGTAA CCTGGAGAGT CACCAACCTG      180
ATGACTGGGC GTACCGCCAA TGCCACCTCC CTCCTGGGAA CTGTACAAGA TGCCTTCCCA      240
AAATTATATT TTGATCTATG TGATCTGGTC GGAGAGGAGT GGGACCCTTC AGACCAGGAA      300

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CCGTATGTCG GGTATGGCTG CAAGTACCCC GCAGGGAGAC AGCGGACCCG GACTTTTGAC	360
TTTTACGTGT GCCCTGGGCA TACCGTAAAG TCGGGGTGTG GGGGACCAGG AGAGGGCTAC	420
TGTGGTAAAT GGGGGTGTGA AACCACCGGA CAGGCTTACT GGAAGCCAC ATCATCGTGG	480
GACCTAATCT CCCTTAAGCG CGGTAACACC CCCTGGGACA CGGGATGCTC TAAAGTTGCC	540
TGTGGCCCCCT GCTACGACCT CTCCAAAGTA TCCAATTCCT TCCAAGGGGC TACTCGAGGG	600
GGCAGATGCA ACCCTCTAGT CCTAGAATTC ACTGATGCAG GAAAAAAGGC TAACTGGGAC	660
GGGCCCCAAT CGTGGGGACT GAGACTGTAC CGGACAGGAA CAGATCCTAT TACCATGTTC	720
TCCCTGACCC GGCAGGTCCT TAATGTGGGA CCCCAGTCC CCATAGGGCC CAACCCAGTA	780
TTACCCGACC AAAGACTCCC TTCCTACCA ATAGAGATTG TACCGGCTCC ACAGCCACCT	840
AGCCCCCTCA ATACCAGTTA CCCCCCTCC ACTACCAGTA CACCCTCAAC CTCCCCTACA	900
AGTCCAAGTG TCCCACAGCC ACCCCCAGGA ACTGGAGATA GACTACTAGC TCTAGTCAAA	960
GGAGCCTATC AGGCGCTTAA CCTCACCAAT CCCGACAAGA CCAAGAATG TTGGCTGTGC	1020
TTAGTGTCGG GACCTCCTTA TTACGAAGGA GTAGCGGTCG TGGGCACTTA TACCAATCAT	1080
TCCACCGCTC CGGCCAACTG TACGGCCACT TCCCAACATA AGCTTACCCT ATCTGAAGTG	1140
ACAGGACAGG GCCTATGCAT GGGGGCAGTA CCTAAACTC ACCAGGCCTT ATGTAACACC	1200
ACCCAAAGCG CCGGCTCAGG ATCCTACTAC CTTGCAGCAC CCGCCGAAC AATGTGGGCT	1260
TGCAGCACTG GATTGACTCC CTGCTTGTC ACCACGGTGC TCAATCTAAC CACAGATTAT	1320
TGTGTATTAG TTGAACTCTG GCCCAGAGTA ATTTACCACT CCCCCGATTA TATGTATGGT	1380
CAGCTTGAAC AGCGTACCAA ATATAAAAGA GAGCCAGTAT CATTGACCCT GGCCCTTCTA	1440
CTAGGAGGAT TAACCATGGG AGGGATTGCA GCTGGAATAG GGACGGGGAC CACTGCCTTA	1500
ATTAAAACCC AGCAGTTTGA GCAGCTTCAT GCCGCTATCC AGACAGACCT CAACGAAGTC	1560
GAAAAGTCAA TTACCAACCT AGAAAAGTCA CTGACCTCGT TGTCTGAAGT AGTCCTACAG	1620
AACCGCAGAG GCCTAGATTT GCTATTCCTA AAGGAGGGAG GTCTCTGCGC AGCCCTAAAA	1680
GAAGAATGTT GTTTTTATGC AGACCACACG GGGCTAGTGA GAGACAGCAT GGCCAAATTA	1740
AGAGAAAGGC TTAATCAGAG ACAAAAACTA TTTGAGACAG GCCAAGGATG GTTCGAAGGG	1800
CTGTTTAATA GATCCCCCTG GTTTACCACC TTAATCTCCA CCATCATGGG ACCTCTAATA	1860
GTA CTCTTAC TGATCTTACT CTTTGACCT TGCATTCTCA ATCGATTGGT CCAATTTGTT	1920
AAAGACAGGA TCTCAGTGGT CCAGGCTCTG GTTTTGACTC AGCAATATCA CCAGCTAAAA	1980



CCCATAGAGT ACGAGCCATG A

2001

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTAGCTAGCT AG

12

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 64 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATATATATAT ATCGATACCA TGGGGCAAAC CGTGACTACC CCTCTGTCCC TCACACTGGC

60

CCAA

64

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 52 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTGATTATGG GCAATTCTTT CCACGTCCTT CCAATGGCCC AGTGTGAGGG AC

52

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 72 base pairs
  - (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGAATTGCCC ATAATCAAAG CGTGGACGTC AAAAAACGCA GGTGGGTGAC ATTTTGTAGC 60  
GCCGAGTGGC CC 72

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 52 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGTTCCATC CCTAGGCCAG CCAACATTGA ATGTGGGCCA CTCGGCGCTA CA 52

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 72 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCCTAGGGA TGGAAC TTTC AATCGCGATC TGATTACTCA AGTGAAAATT AAAGTGTTC A 60  
GCCCCGGACC CC 72

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 52 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTGACAATAT AAGGAAC TTG ATCGGGATGG CCGTGGGGTC CGGGGCTGAA CA 52

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTTCCTTAT ATTGTCACAT CGGAGGCTCT CGCTTTCGAT CCACCACCTT GGGTGAAACC 60

ATTCGTGCAT CC 72

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22

AGGAGCGCTG GGTGGGAGGG GTGGAGGTGG TTTGGGATGC ACGAATGGTT TC 52

(2) INFORMATION FOR SEQ ID NO:23

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCCACCCA GCGCTCCTAG CCTGCCCTTG GAGCCCCAC GAAGCACACC ACCCAGGAGC 60

AGCTTG TACC CT 72

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTTTAGGTTT GGCGCCGAGG CTGGGGGTCA GAGCAGGGTA CAAGCTGCTC CT 52

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATATATATAT ATCGATACC 19

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTTTAGGTTT GGCGCCGAGG 20

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGGAGTGGTA ACAGTCTGGC CTTAATTCTC AG

32

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGTCGACCT CGAGAATTAA TAC

23

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTGGGAGACG TCCCAGGGAC TTC

23

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGCCAGACTG TTACCACTCC CTGAAGTTTG AC

32

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CATCGATAAA ATAAAAGATT TTATTTAGTC

30

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CAAATGAAAG ACCCCCGCTG AC

22

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTATGAGCT CGCCTTCTAG TTGCCAGC

28

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCTATGAATT CGCGCCGCC ATAGAGCCCA CCGCATCC

38

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TATATATGAG CTCTAATAAA ATGAGGAAAT TGCATCGCAT TGTC

44

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTATGAATT CGCGCCGCA TAGAATGACA CCTACTCAGA CAATGCGA

48

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCTCGTTTAG TGAACGTCA G

21

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 29 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TATCCGAGCT CATGGCTCGT ACTCTATGG

29

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 37 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CACCTATGCT AGCCACCATG GCGCGTTCAA CGCTCTC

37

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 36 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CACCTATGCG GCCGCTCATG GCTCGTACTC TATGGG

36

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 39 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CACCTATGCG GCCGCCACCA TGGCGCGTTC AACGCTCTC 39

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 429 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ATCGATACCA TGGGGCAAAC CGTGACTACC CCTCTGTCCC TCACACTGGG CCATTGGAAG	60
GACGTGGAAA GAATTGCCCA TAATCAAAGC GTGGACGTCA AAAAACGCAG GTGGGTGACA	120
TTTTGTAGCG CCGAGTGGCC CACATTCAAT GTTGGCTGGC CTAGGGATGG AACTTTCAAT	180
CGCGATCTGA TTA CTCAAGT GAAAATTAAG GTGTTTCAGCC CCGGACCCCA CGGCCATCCC	240
GATCAAGTTC CTTATATTGT CACATGGGAG GCTCTCGCTT TCGATCCACC ACCTTGGGTG	300
AAACCATTCG TGCATCCCAA ACCACCTCCA CCCCTCCCAC CCAGCGCTCC TAGCCTGCCC	360
TTGGAGCCCC CACGAAGCAC ACCACCCAGG AGCAGCTTGT ACCCTGCTCT GACCCCCAGC	420
CTCGGCGGCC	429

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CGAATCGATA CCATGGGCCA GACTGTTACC AC 32

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 23 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:  
CATTCTGCAG AGCAGAAGGT AAC

23

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 14 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:  
TAAGCGGCCG CTTA

14

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### Claims

We claim:

1. A retroviral vector construct comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein said vector construct contains *gag/pol* coding sequences which have been modified to contain two or more stop codons.
2. The retroviral vector construct according to claim 1 wherein said vector construct lacks an extended packaging signal.
3. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral nucleic acid sequence upstream of said 5' LTR.
4. The retroviral vector construct according to claim 3 wherein said construct lacks an *env* coding sequence upstream of said 5' LTR.
5. The retroviral vector construct according to claim 1 wherein said construct lacks an *env* coding and/or untranslated *env* sequence upstream of said 3' LTR.
6. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral packaging signal sequence downstream of said 3' LTR.
7. The retroviral vector construct according to claim 1 wherein said retrovector is constructed from a retrovirus selected from the group consisting of amphotropic, ecotropic, xenotropic or polytropic viruses.
8. The retroviral vector construct according to claim 1 wherein said retrovector is constructed from a Murine Leukemia Virus.
9. The retroviral vector construct according to claim 1, further comprising a heterologous sequence.
10. The retroviral vector construct according to claim 9 wherein said heterologous sequence is a gene encoding a cytotoxic protein.

11. The retroviral vector construct according to claim 10 wherein said cytotoxic protein is selected from the group consisting of ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A.

12. The retroviral vector construct according to claim 9 wherein said heterologous sequence is an antisense sequence.

13. The retroviral vector construct according to claim 9 wherein said heterologous sequence encodes an immune accessory molecule.

14. The retroviral vector construct according to claim 13 wherein said immune accessory molecule is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13 and IL-15.

15. The retroviral vector construct according to claim 13 wherein said immune accessory molecule is selected from the group consisting of ICAM-1, ICAM-2, b-microglobulin, LFA3, HLA class I and HLA class II molecules.

16. The retroviral vector construct according to claim 9 wherein said heterologous sequence encodes a gene product that activates a compound with little or no cytotoxicity into a toxic product.

17. The retroviral vector construct according to claim 16 wherein said gene product is selected from the group consisting of HSVTK, VZVTK and cytosine deaminase.

18. The retroviral vector construct according to claim 9 wherein said heterologous sequence is a ribozyme.

19. The retroviral vector construct according to claim 9 wherein said heterologous sequence is a replacement gene.

20. The retroviral vector construct according to claim 19 wherein said replacement gene encodes a protein selected from the group consisting of Factor VIII, ADA, HPRT, CF and the LDL Receptor.

21. The retroviral vector construct according to claim 9 wherein said heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

22. A producer cell line, comprising a *gag/pol* expression cassette, an *env* expression cassette and a retroviral vector construct, wherein a 3' terminal end of a *gag/pol* gene encoded within said *gag/pol* expression cassette lacks homology with a 5' terminal end of an *env* gene encoded within said *env* expression cassette, and wherein a 3' terminal end of said *env* gene lacks homology with said retroviral vector construct, with the proviso that said retroviral vector construct overlaps with at least 4 nucleotides of a 5' terminal end of said *gag/pol* gene encoded within said *gag/pol* expression cassette.

23. The producer cell line according to claim 22 wherein said retroviral vector construct is a retroviral vector construct according to any one of claims 1 to 21.

24. The producer cell line according to claim 22 wherein said *gag/pol* expression cassette comprises a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *gag/pol* gene has been deleted without affecting the biological activity of integrase.

25. The producer cell line according to claim 24 wherein said 3' terminal end has been deleted upstream of nucleotide 5751 of Sequence ID No. 1.

26. The producer cell line according to claim 24 wherein said promoter is a heterologous promoter.

27. The producer cell line according to claim 24 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.



28. The producer cell line according to claim 24 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

29. The producer cell line according to claim 28 wherein said heterologous polyadenylation sequence is selected from the group consisting of the SV40 late poly A signal, the SV40 early poly A signal and a bovine growth hormone poly A signal.

30. The producer cell line according to claim 22 wherein said *env* expression cassette comprises a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 consecutive retroviral nucleotides are included upstream of said *env* gene.

31. The producer cell line according to claim 22 wherein said *env* expression cassette comprises a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein said *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene.

32. The producer cell line according to claim 22 wherein said *env* expression cassette comprises a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *env* gene has been deleted without effecting the biological activity of *env*.

33. The producer cell line according to claim 32 wherein a 3' terminal end of said *env* gene has been deleted such that a complete R peptide is not produced by said expression cassette.

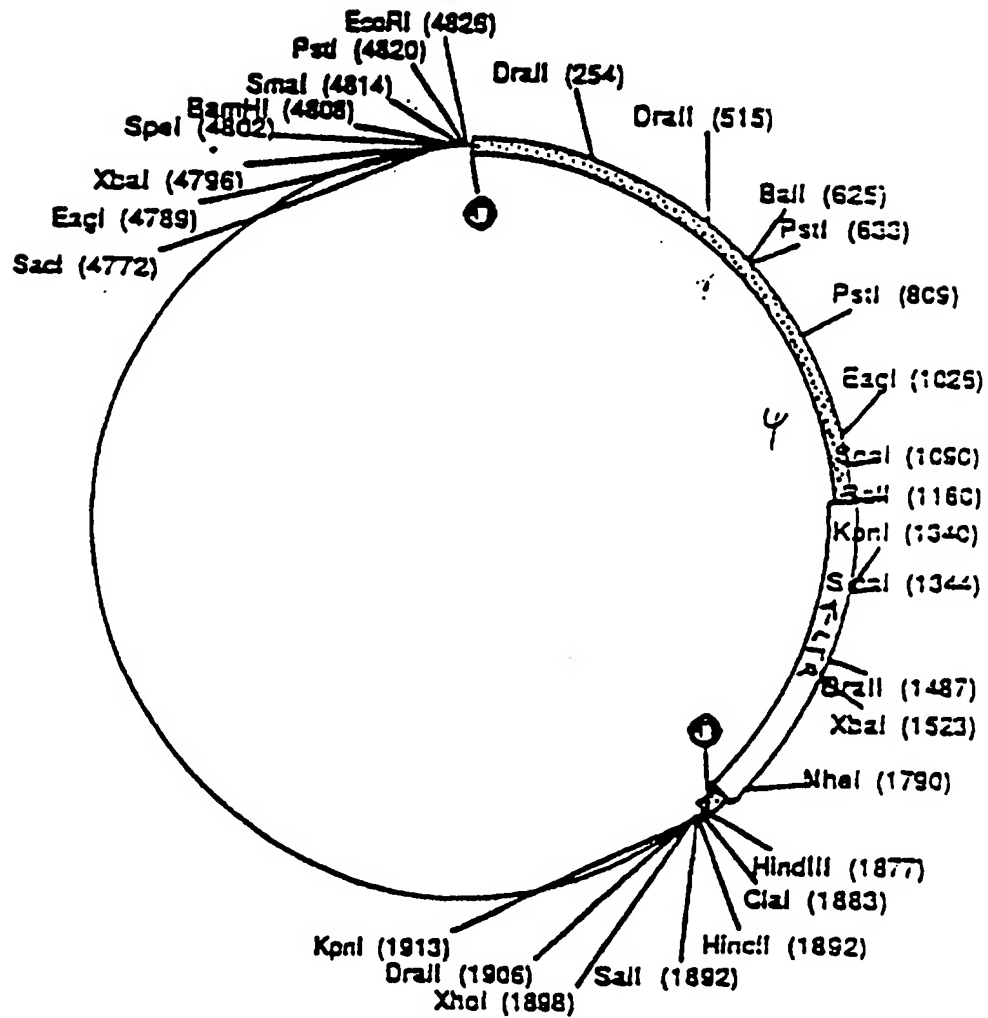
34. The producer cell line according to claim 32 wherein said *env* gene is derived from a type C retrovirus, and wherein the 3' terminal end has been deleted such that said *env* gene includes less than 18 nucleic acids which encode said R peptide.

35. The producer cell line according to claim 32 wherein said promoter is a heterologous promoter.

36. The producer cell line according to claim 35 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.

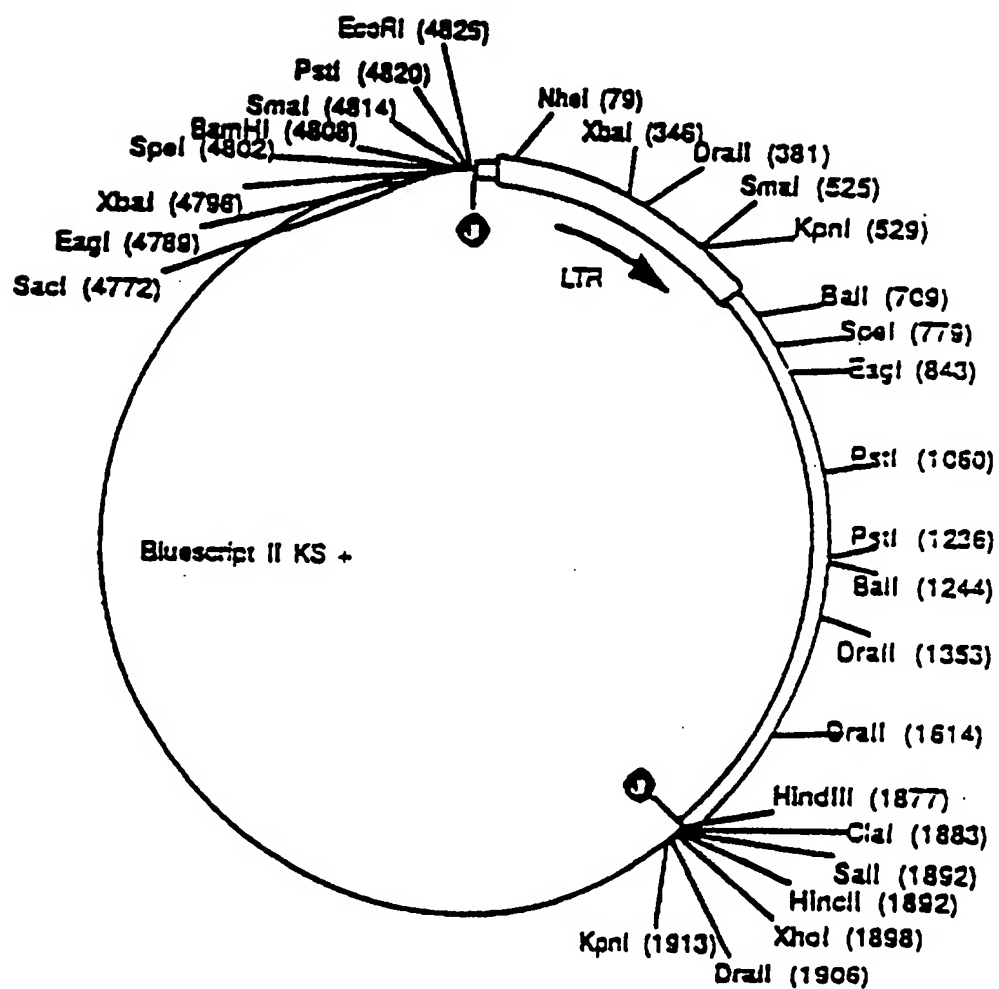
37. The producer cell line according to claim 32 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

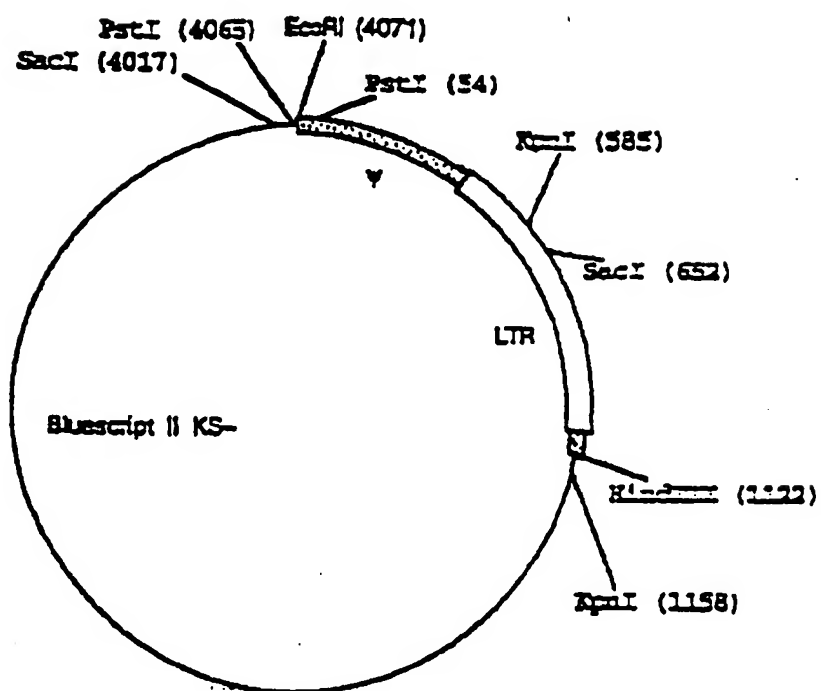
38. The producer cell line according to claim 37 wherein said heterologous polyadenylation is selected from the group consisting of the SV40 late poly A signal, the SV40 early poly A signal and a bovine growth hormone polyadenylation sequence.

**FIGURE 1****pKS2+Eco57I-LTR(+)**

## FIGURE 2

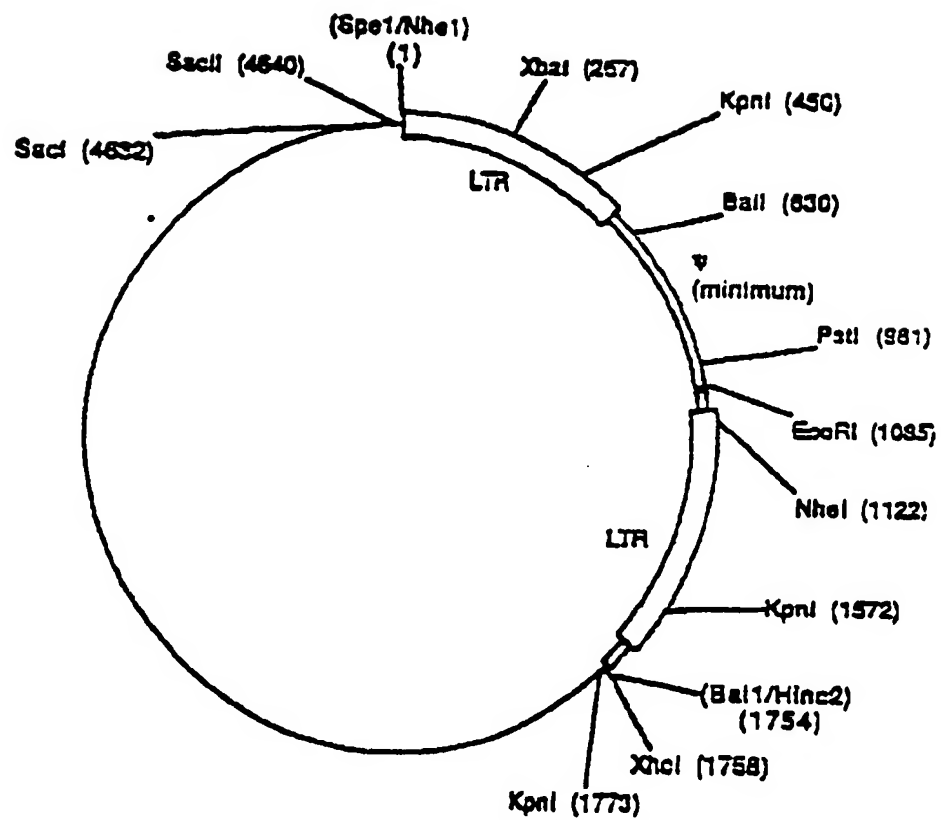
pKS2+Eco57I-LTR(-)



**FIGURE 3****pKS2+LTR-EcoRI**

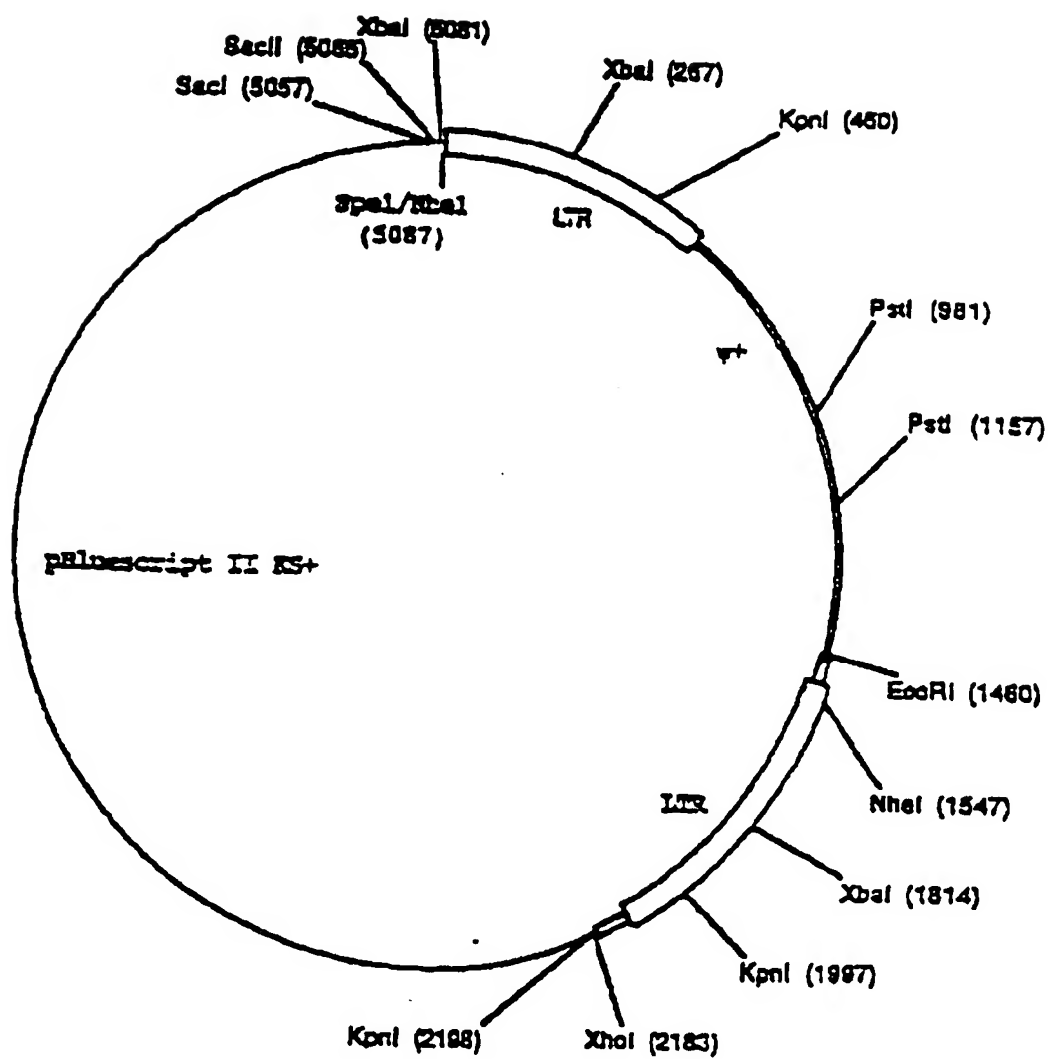
## FIGURE 4

pR1



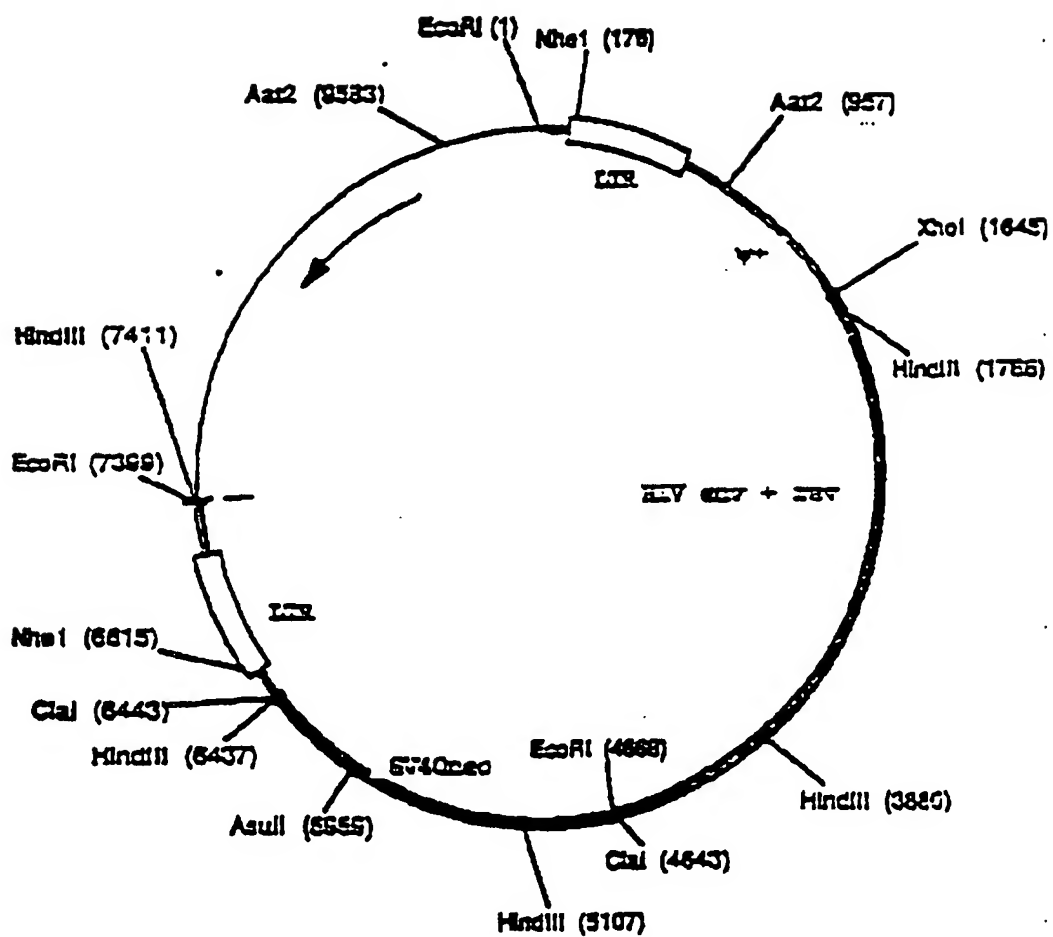
## FIGURE 5

pR2



## FIGURE 6

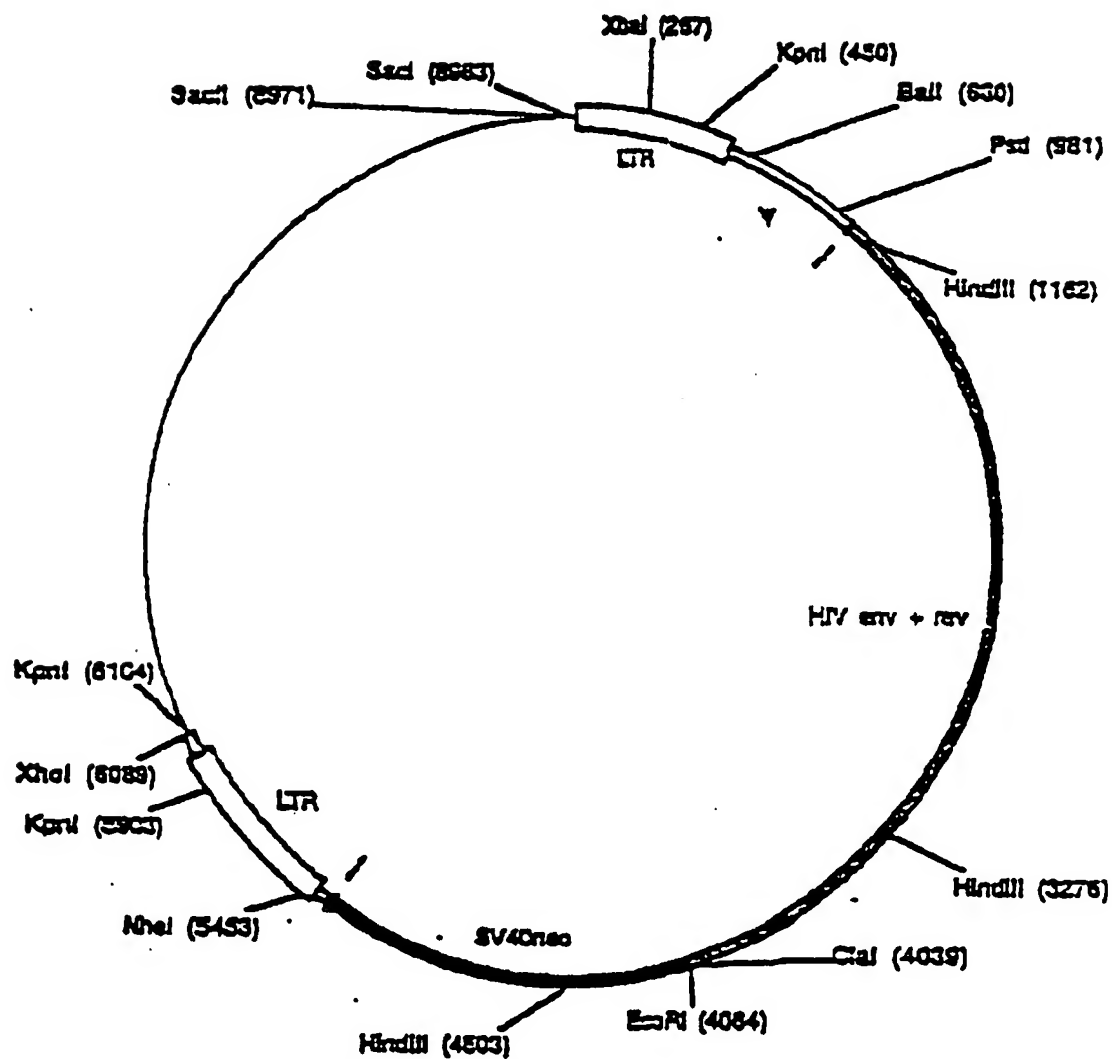
pKT1





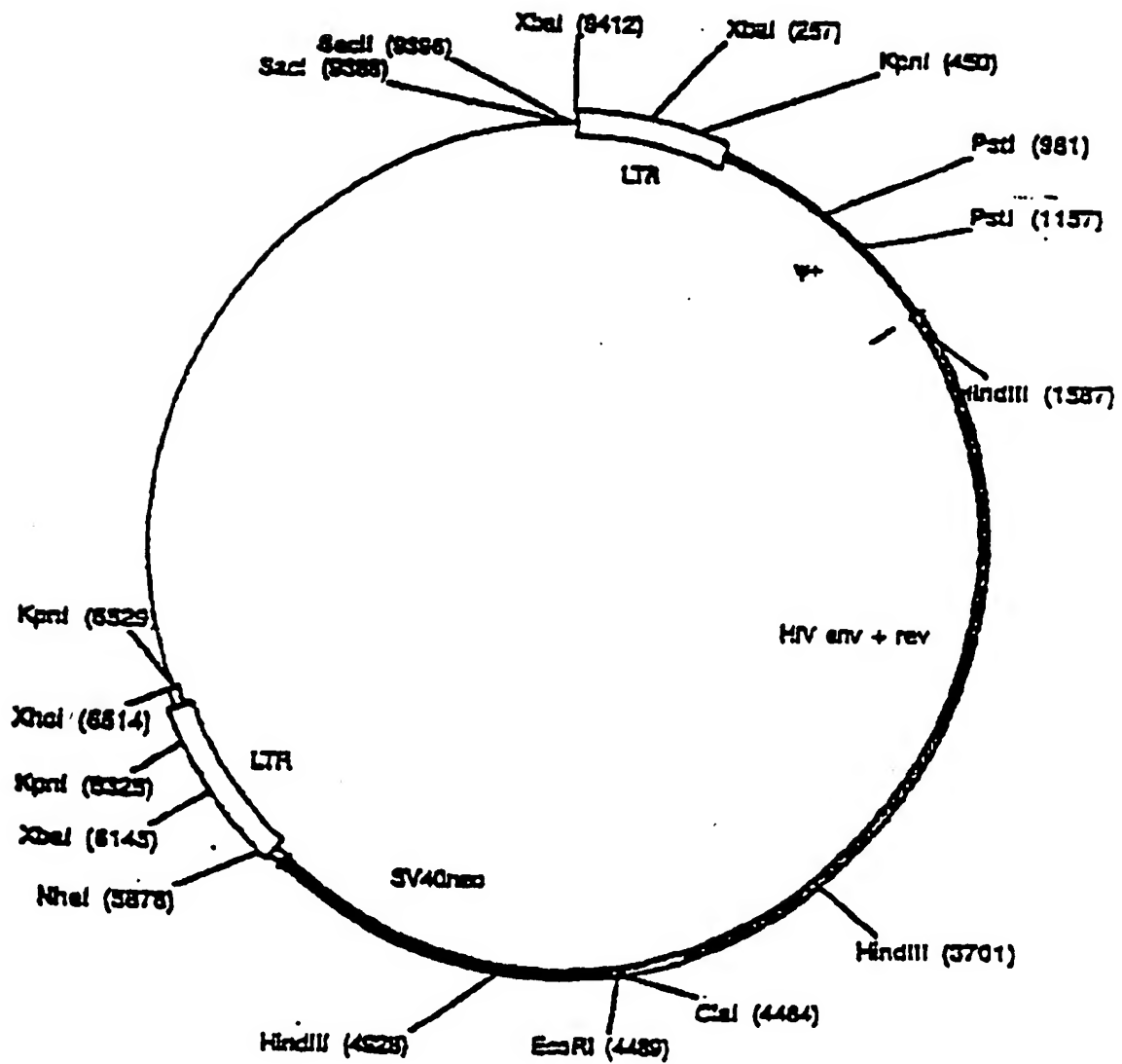
## FIGURE 7

pR1-HIVenv



## FIGURE 8

pR2-HIVenv



## FIGURE 9

Prewobble Sequence for  
MoMLV *Gag/Pol*

```

1  ATG  GGC  GCG  ACT  GGT  ACC  ACT  GGC  TTA  AGT  TTG  ACC  TTA  GGT  GGC  TGG  AAA
19>Met  Gly  Gln  Thr  Val  Thr  Thr  Pro  Leu  Ser  Leu  Thr  Leu  Gly  His  Trp  Lys

52  GAT  GTC  GCG  CGG  ATC  GGT  GAC  AAC  CAG  TCG  GGA  GAT  GTC  AAG  AAG  AAG  CGT
19>Asp  Val  Glu  Arg  Ile  Ala  His  Asn  Gln  Ser  Val  Asp  Val  Lys  Lys  Arg  Arg

103 TGG  GGT  ACC  TTC  TGC  TGT  GGA  GAA  TGG  CCA  ACC  TTT  AAC  GTC  GGA  TGG  CCG
35>Trp  Val  Thr  Phe  Cys  Ser  Ala  Glu  Trp  Pro  Thr  Phe  Asn  Val  Gly  Trp  Pro

154 CCA  GGC  GGC  ACC  TTT  AAC  CCA  GGC  CAC  ATC  ACC  CCG  GGT  AAG  ATC  AAG  GTC
52>Arg  Asp  Gly  Thr  Phe  Asn  Arg  Asp  Leu  Ile  Thr  Gln  Val  Lys  Ile  Lys  Val

205 TTT  TCA  GGT  GGC  CCG  CAT  GGA  CAC  CCA  GAC  CAG  GTC  CCG  TAC  ATC  GGC  ACC
59>Phe  Ser  Pro  Gly  Pro  His  Gly  His  Pro  Asp  Gln  Val  Pro  Tyr  Ile  Val  Thr

256 TGG  GAA  GGC  TTG  GGT  TTT  GAC  CCG  CTT  CCG  TGG  GTC  AAG  CCG  TTT  GGA  GGC
86>Trp  Glu  Ala  Leu  Ala  Phe  Asp  Pro  Pro  Pro  Trp  Val  Lys  Pro  Phe  Val  His

307 CCT  AAG  CGT  CCG  CTT  GGT  CTT  CTT  CCA  TCC  GGC  CCG  TCT  CTC  CCG  CTT  GAA
103>Pro  Lys  Pro  Pro  Pro  Pro  Leu  Pro  Pro  Ser  Ala  Pro  Ser  Leu  Pro  Leu  Glu

358 GGT  GGT  GGT  TGG  ACC  CCG  GGT  GGA  TCC  TCC  CTT  TTT  CCA  GGC  CAC  ACT  CTT
120>Pro  Pro  Arg  Ser  Thr  Pro  Pro  Arg  Ser  Ser  Leu  Tyr  Pro  Ala  Leu  Thr  Pro

      Nari (415)
409 TCT  CCA  GGC  GGC
137>Ser  Leu  Gly  Ala

```

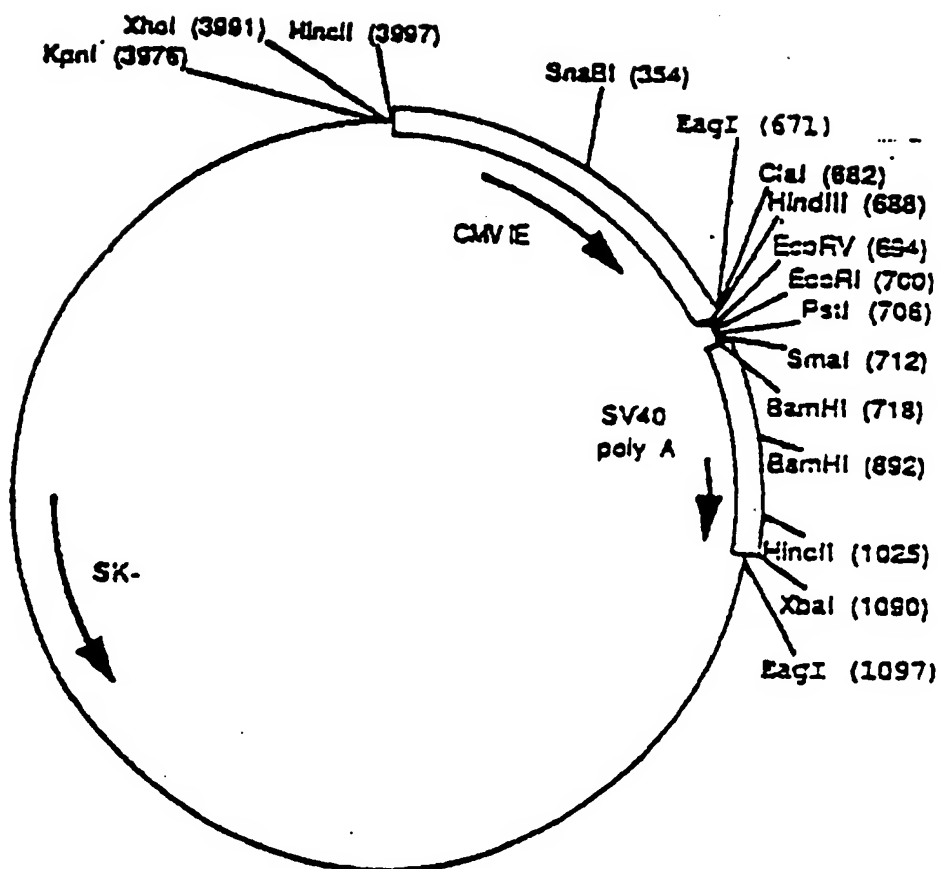
## FIGURE 10

Wobble Sequence for  
MoMLV Gag/Pol

A TAT ATA TAT ATC GAT ACC ATG GGG GGA ACC GGG ACT ACC GGT GGG TGG  
 ▶ Met Gly Gln Thr Val Thr Thr Pro Leu Ser  
 CTC ACA CCG GGC CAT TGG AAG GGC GGG GGA AGA ATT GGC CAT AAT GGA ACC  
 ▶ Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser  
 GGG GGC GGC AAA AAA GGC AGG TGG GGG ACA TTT TGT AGC GGC GGC TGG GGC  
 ▶ Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro  
 ACA TTC AAT GAT GGC TGG GGT AAG GAT GGA ACT TTC AAT GGC GAT GGG ATT  
 ▶ Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile  
 ACT GGA GGG AAA ATT AAA GGG TTC AGC GGC GGA GGC GGC GGC GGC GGC  
 ▶ Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp  
 GGA GGT GGT TAT ATT GGC ACA TGG GGG GGT GGC GGT TTC GAT GGA GGA GGT  
 ▶ Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro  
 TGG GGG AAA GGA TTC GGG CAT GGC AAA GGA GGT GGA GGC GGC GGC GGC AGC  
 ▶ Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser  
 GGT GGT AGC GGG GGC TGG GGG GGC GGA GGA AGC AGC GGA GGC AGC AGC AGC  
 ▶ Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser  
 Nari  
 TGG TGC GGT GGT GGG ACC GGC AGC GTC GGC GGC AAA GGT AAA C  
 ▶ Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ????????

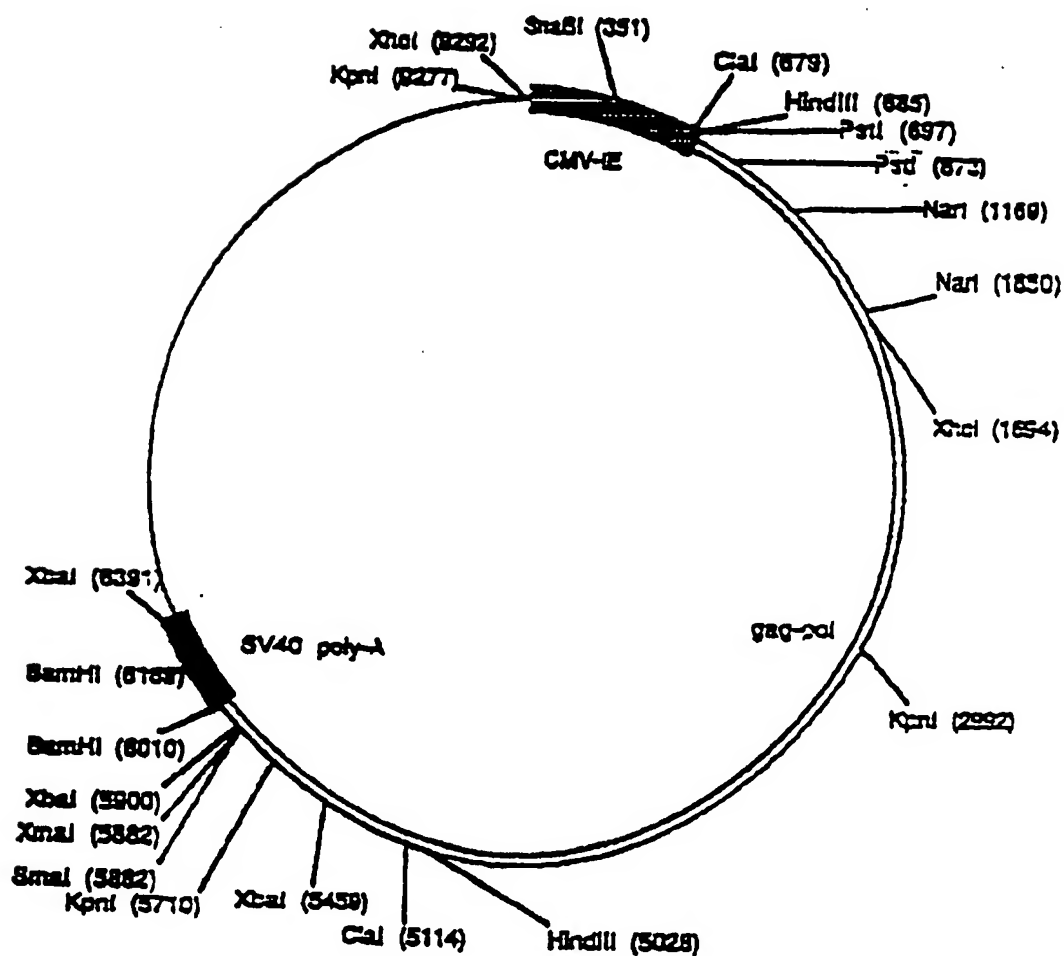
## FIGURE 11

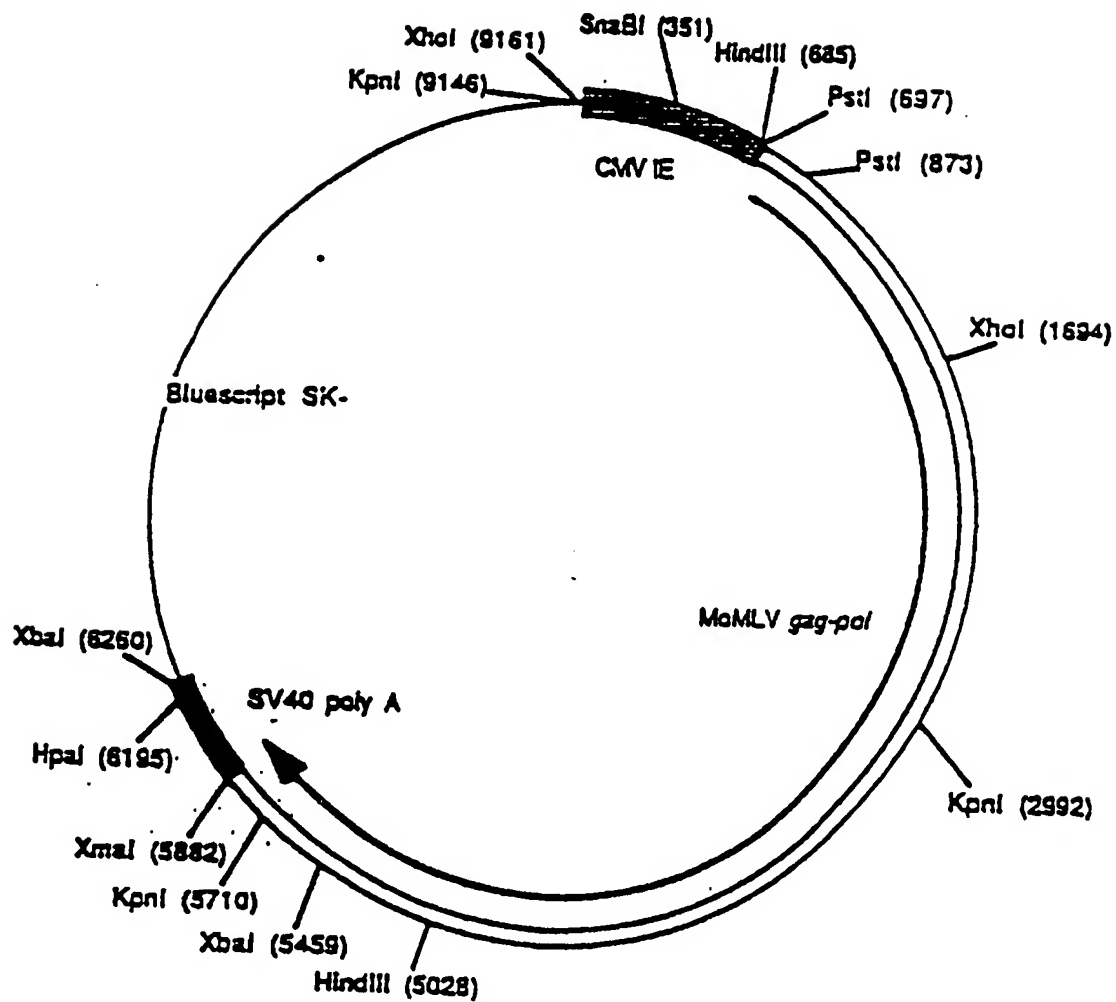
pHCMV-PA



## FIGURE 12

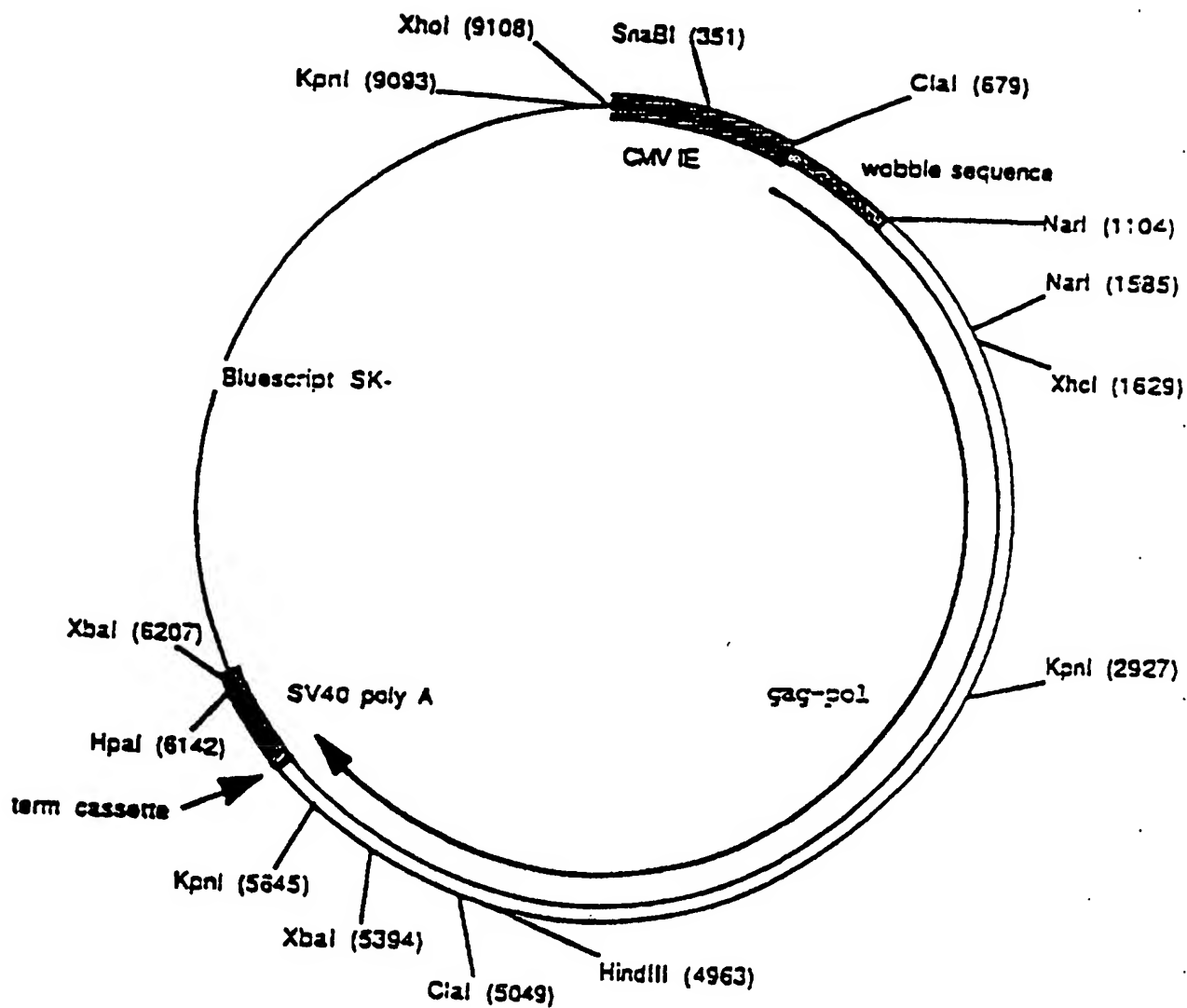
pCMV Gag/Pol



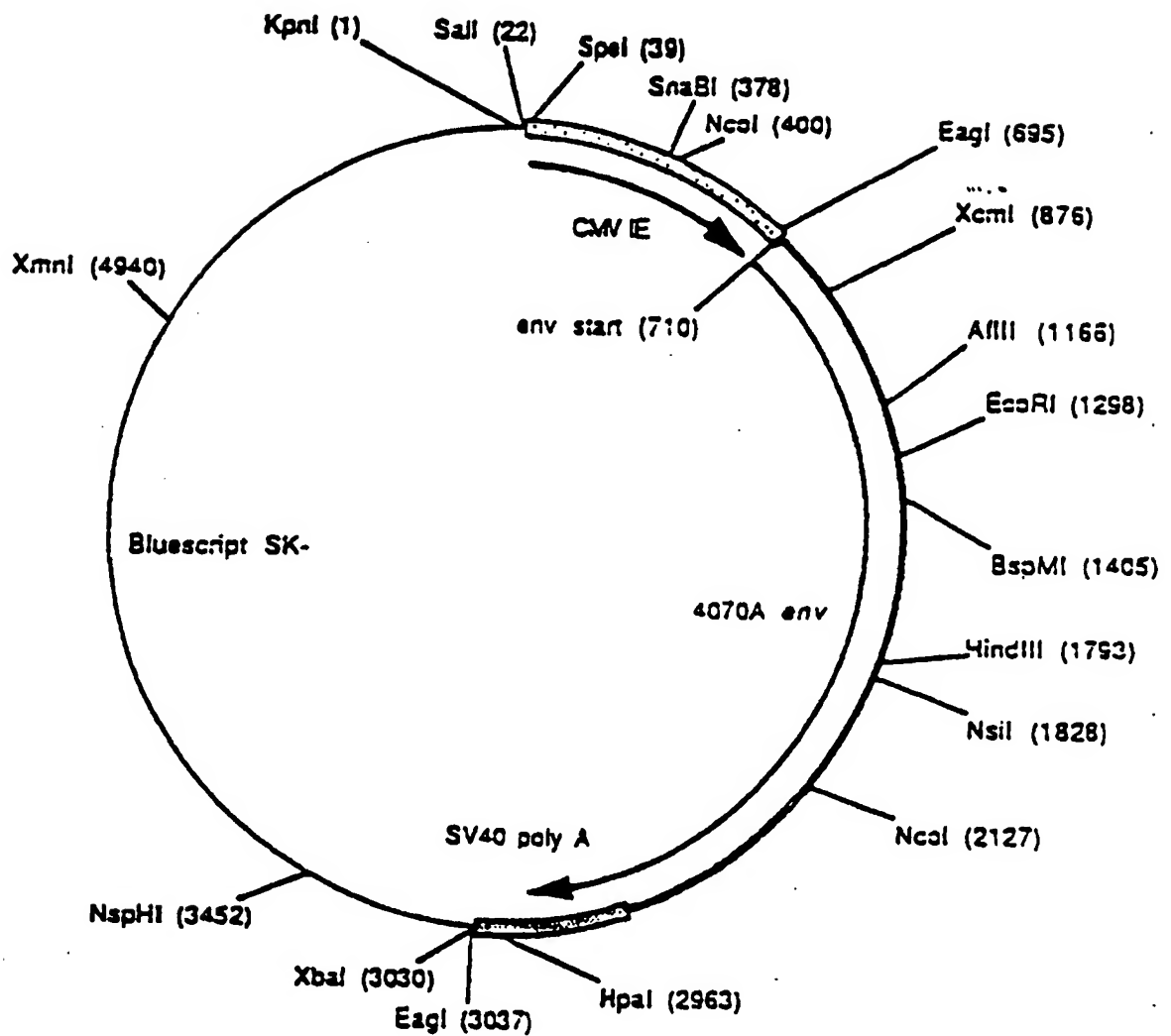
**FIGURE 13****pCMVgpSma**

## FIGURE 14

pCMVgp-X

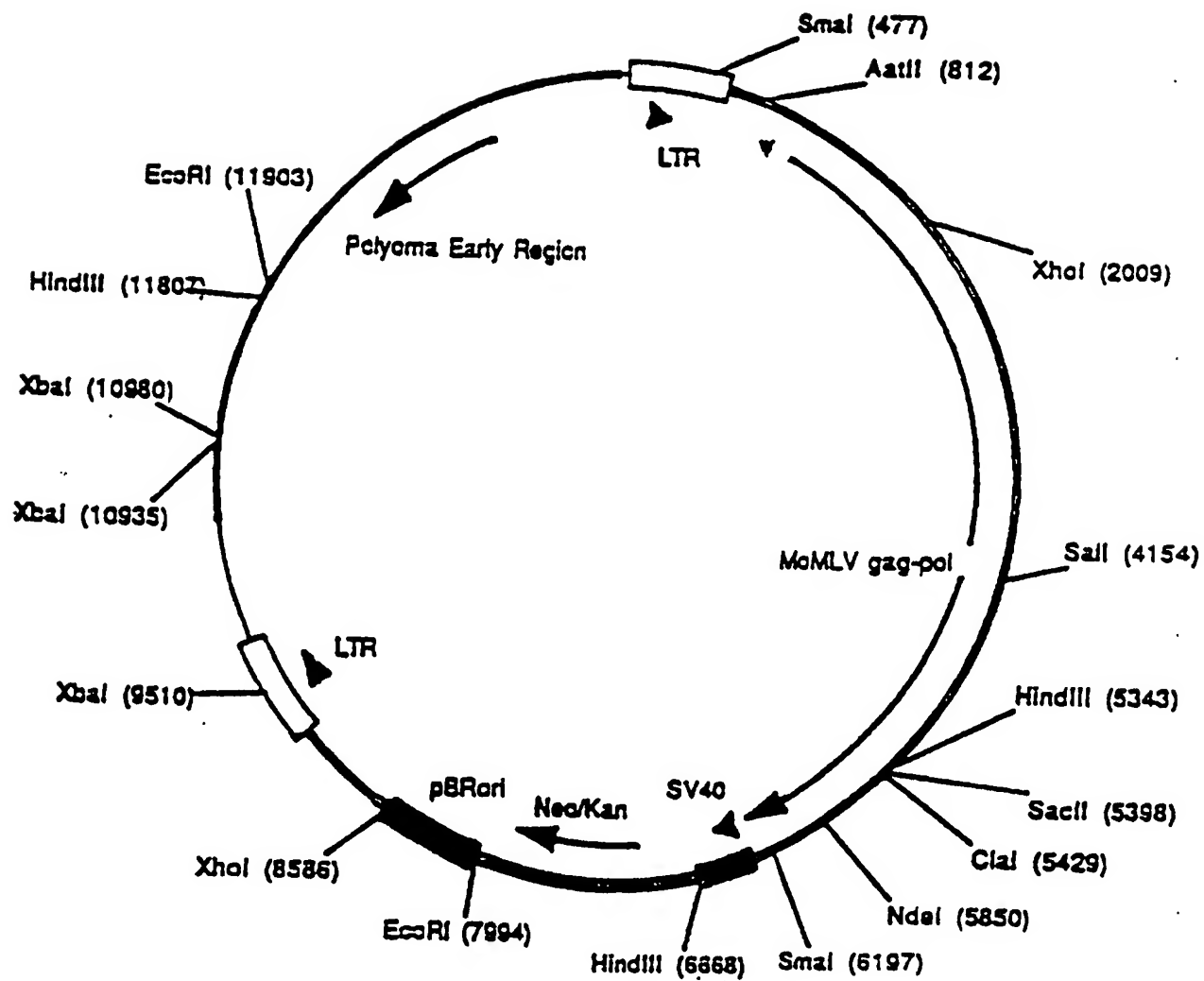




**FIGURE 15****pCMV env-X**

## FIGURE 16

pRgpNeo



## Figure 17A

VIRUS	SPECIES OF ISOLATION	TYPE <sup>1</sup>
AEV (avian erythroblastosis virus)	chicken	C.X.T
ALV (avian leukosis virus)	chicken	C.N or X.N
AMV (avian myeloblastosis virus)	chicken	C.X.T
ASV (avian sarcoma virus)	chicken	C.X.T
BaEV (baboon endogenous virus)	baboon ( <i>Papio spp.</i> )	C.N.N
BILN	<i>P. hamadryas</i>	
M7	<i>P. cynocephalus</i>	
M28	<i>P. cynocephalus</i>	
PP-1-Lu	<i>P. papio</i>	
TG-1-K	gelada	
BLV (bovine leukemia virus)	cow	C.X.N
BSV (bovine syncytial virus)	cow	S.X.N
CAEV (caprine arthritis-encephalitis virus)	goat	L.X.N
CERV-CI. CERV C-II	<i>Mus cervicolor</i>	C.N.N
CCC	cat	C.N.N
CPC-1	colobus monkey	C.N.N
CSRV (corn snake retrovirus)	corn snake	C.
CSV (chick syncytial virus)	chicken	C.X.N
DLAV (duck infectious anemia virus)	duck	C.X.N
DKV (deer kidney virus)	black-tailed deer	C.N.N
DPC-1	agouti	C.N.N
ELAV (equine infectious anemia virus)	horse	C.X.N
ESV (Esh sarcoma virus)	chicken	C.X.T
FeLV (feline leukemia virus)	cat	C.N or X.N
FeSV (feline sarcoma virus)	cat	C.X.T
GA (Gardner-Amstein)		
SM (McDonough)		
ST (Snvder-Theilen)		
FS-1	<i>Felis sylvestris</i> (wildcat)	C.N.N
FSFV (feline syncytium-forming virus)	cat	S.X.N
FuSV (Fujinami sarcoma virus)	chicken	C.X.T
GALV (gibbon ape leukemia virus)	gibbon	C.X.N
GLV (goat leukoencephalitis virus)	see CAEV	
GPV (golden pheasant virus)	golden pheasant	C.N.N
HaLV (hamster leukemia virus)	hamster	C.N.N
IVL (induced leukemia virus)	chicken	C.N.N
LLV (lymphoid leukosis virus)	see ALV	
LPDV (lymphoproliferative disease of turkeys)	turkey	C.X.T
M432	<i>Mus cervicolor</i>	B.N.N
M832	<i>Mus caroli</i>	B.N.N

<sup>1</sup> The first letter denotes classification: (B) B-type oncovirus; (C) C-type oncovirus; (D) D-type oncovirus; (L) lentivirus; (S) spumavirus. The second letter denotes origin: (N) endogenous; (X) exogenous; (R) recombinant. The third letter denotes ability to induce morphological transformation: (T) transforming (i.e., containing an onc sequence); (N) nontransforming; (?) unknown.

Figure 17B

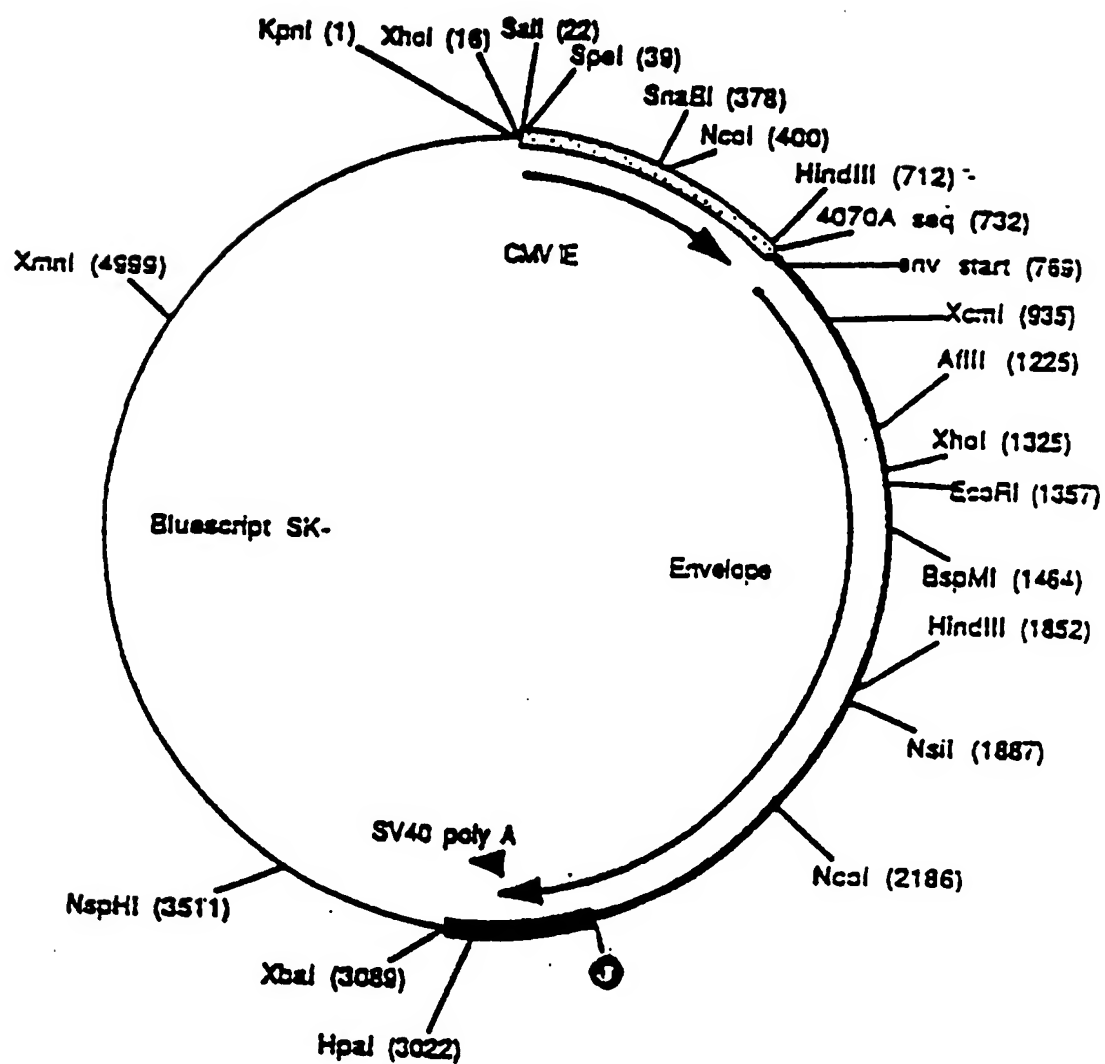
MAC-1	stumptail monkey	C.N.N
Maedi	sheep	L.X.N
MAV (mveloblastosis-associated virus)	chicken	C.X.N
MC29 (mvelocytomatosis virus)	chicken	C.X.T
MCF (mink cell focus-inducing virus)	mouse	C.N.R.N
MH2 (mvelocytomatosis virus)	chicken	C.X.T
MILV (mink leukemia virus)	mink	C.N.N
MLV (murine leukemia virus)	mouse	C.X or N.N
Ab (Abelson)		C.X.T
Fr (Friend)		C.X.N
Graff		C.X.N
Gross		C.N.N
Ki (Kirsten)		C.X.N
Mo (Moloney)		C.X.N
Ra (Rauscher)		C.X.N
MMC-1	rhesus monkey	C.N.N
MMTV (mouse mammary tumor virus)	mouse	B.X or N.N
MPMV (Mason-Pfizer monkey virus)	rhesus monkey	D.X.N
MSV (murine sarcoma virus)	mouse	C.X.T
BALB		
FBJ (Finkel-Biskis-Jenkins)		
FBR		
Gz (Gazdar)		
Ha (Harvey)		
Ki (Kirsten)		
Mo (Moloney)		
MPV <sup>1</sup> (mveloproliferative)		
OS2 (osteosarcoma)		
MyLV (mveloid leukemia)	mouse	C.X.N
OK10 (mvelocytomatosis virus)	chicken	C.X.T
OMC-1	owl monkey	C.N.N
PK-15	pig	C.N.N
PO-1-Lu	langur	D.N.N
PPV (progressive pneumonia virus)	sheep	L.X.N
PRCII, PRCIV (Poultry Research Centre)	chicken	C.X.T
R-35	rat	C.X?,T
RaLV (rat leukemia virus)	rat	C.X.N
RaSV (rat sarcoma virus)	rat	C.X.T
RAV-n (Rous-associated virus)	see ALV	
RAV-0 (Rous-associated virus 0)	chicken	C.N.N
RAV-60 (Rous-associated virus 60)	chicken	C.R.N
RAV-61 (Rous-associated virus 61)	ring-necked pheasant	C.R.N
RD114	cat	C.N.N
REAV (reticuloendotheliosis-associated virus)	turkey	C.X.N

Figure 17C

REV (reticuloendotheliosis virus)	birds	C,X,N
REV-T (reticuloendotheliosis virus-transforming)	turkey	C,X,T
RIE (Rous interference factor)	see ALV	
RPL-n (Regional Poultry Laboratory)	see ALV	
RPV (ring-necked pheasant virus)	ring-necked pheasant	C,R,N
RSV (Rous sarcoma virus)	chicken	C,X,T
B77 (Bratislava)		
BH (Brvan high titer)		
BS (Brvan standard)		
CZ (Carr-Zilber)		
EH (Engelbreth-Holm)		
HA (Harris)		
PR (Prague)		
SR (Schmidt-Ruppin)		
SFV-n (simian foamy virus)	monkey	S,X,N
SFFV (spleen focus-forming virus)	mouse	C,X, or R,N or T
Friend		
MPV		
Rauscher		
SiSV (simian sarcoma virus)	see SSV	
SLV (simian lymphoma virus)	see GALV	
SMRV (squirrel monkey retrovirus)	squirrel monkey	D,N,N
SMV (simian myelogenous leukemia virus)	see GALV	
SSAV (simian sarcoma-associated virus)	woolly monkey	C,X,N
SSV (simian sarcoma virus)	woolly monkey	C,X,T
TRV-1	tree shrew	C,N,N
UR-n (University of Rochester)	chicken	C,X,T
Vand C-1	tree mouse	C,N,N
Visna	sheep	L,X,N
VRV (viper retrovirus)	Russell's viper	C,N,?
WMV (woolly monkey virus)	see SSV	
WoLV (woolly monkey leukemia virus)	see SSAV	
Y73 (Yamazuchi 73)	chicken	C,X,T

## FIGURE 18

## CMV EnvAm-Eag-X-less



**FIGURES 19A & 19B****19A**

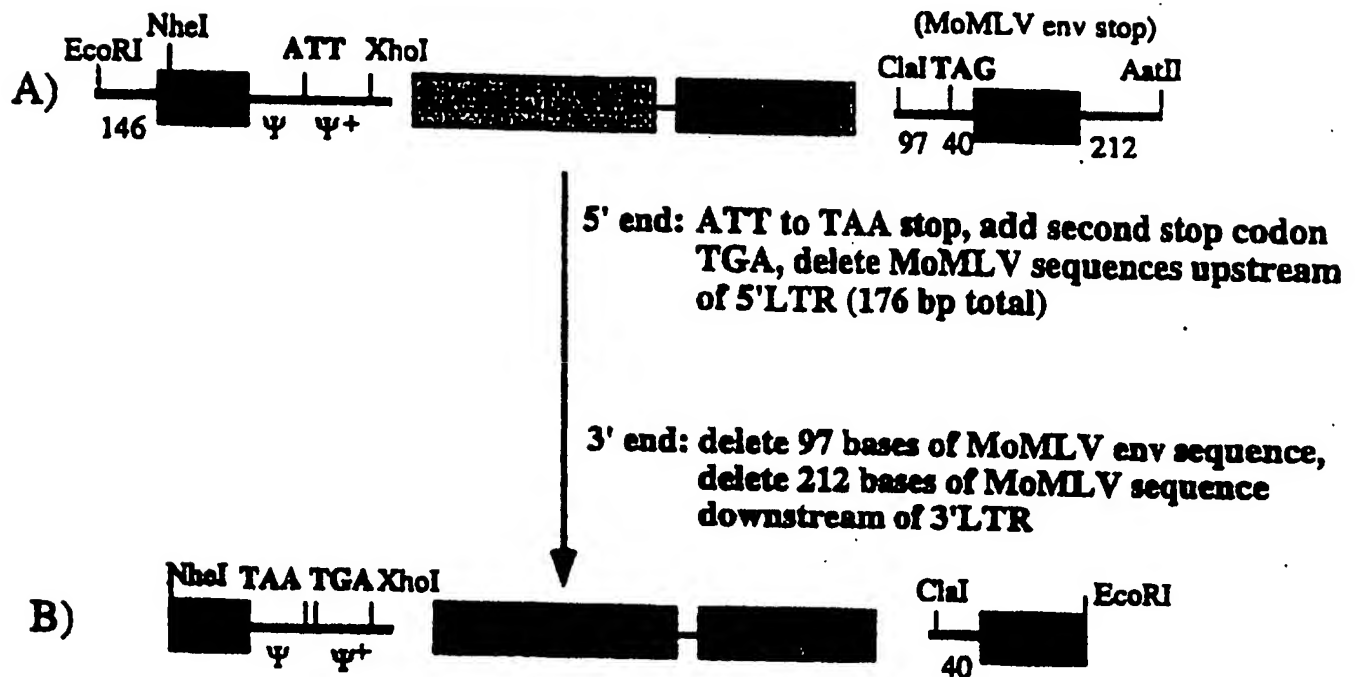
**CMV Promoter** — wobble gag — SVneo — **LTR**

**19B**

**CMV Promoter** — normal gag — SVneo — **LTR**

## Figure 20

### RETROVIRAL BACKBONE (N2-derived)



### CROSS-LESS RETROVIRAL BACKBONE: pBA-5

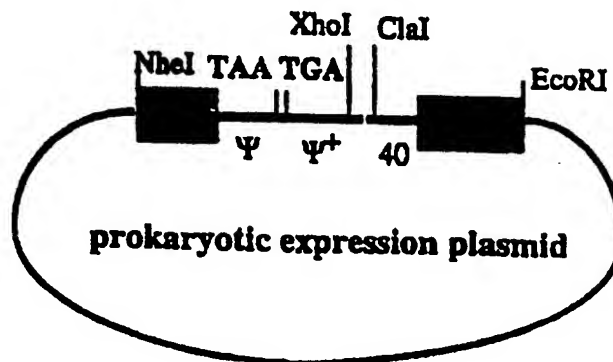
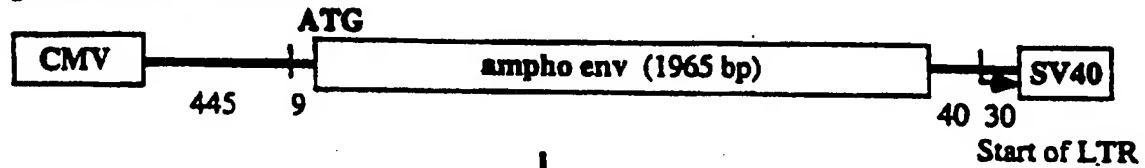




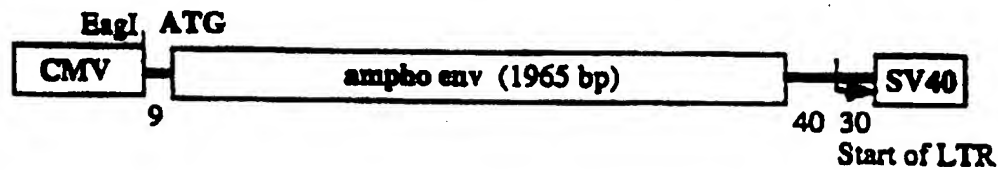
Figure 21

# AMPHOTROPIC ENVELOPE CONSTRUCTS

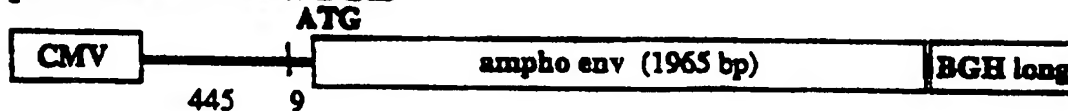
pCMVenvamDraI



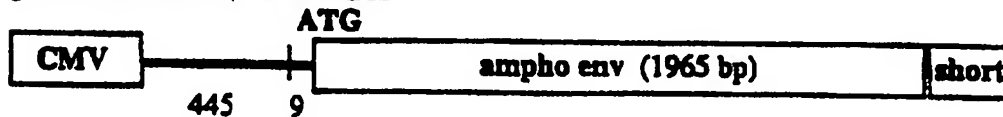
pCMVenvam.x



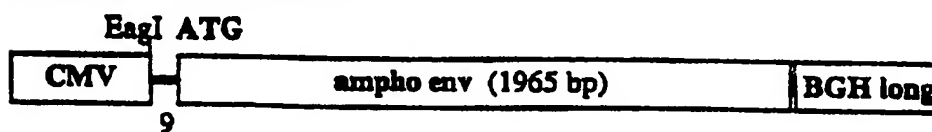
pCMVenvamDraI/BGH



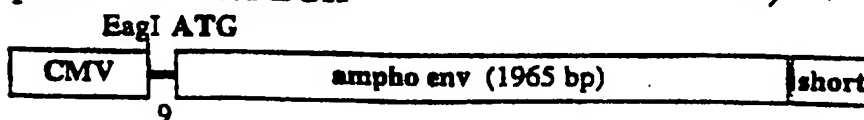
pCMVenvamDraI/sBGH



pCMVenvam.x/BGH



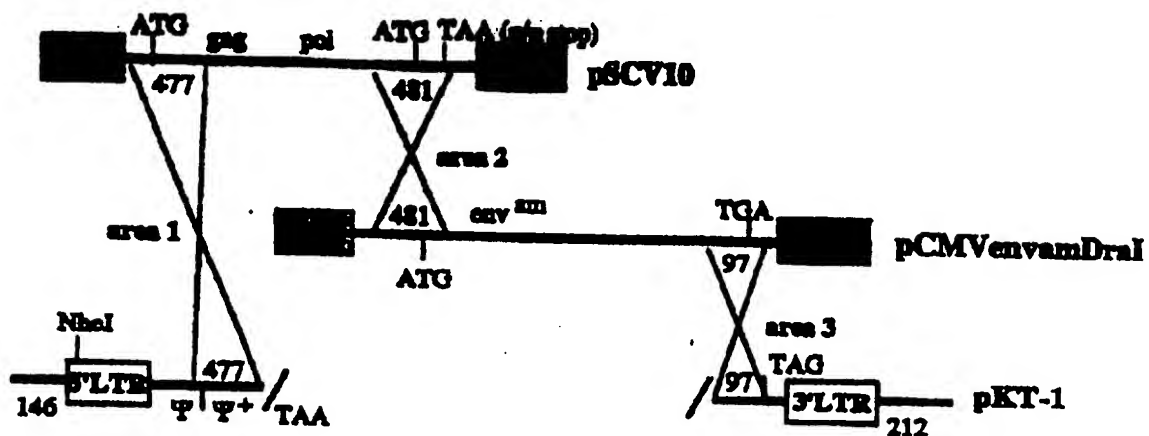
pCMVenvam.x/s BGH



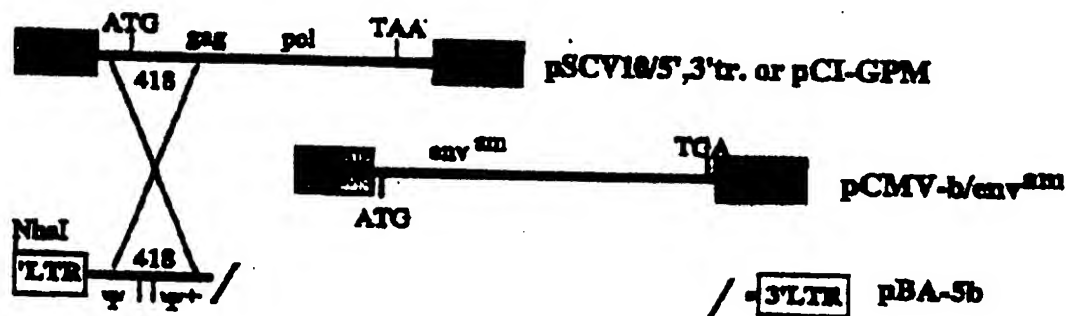
23/24

# FIGURE 22

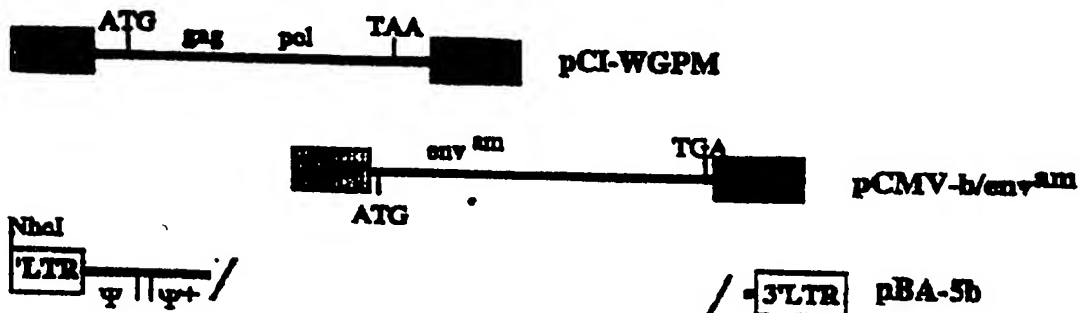
## A. Unmodified retroviral components (three areas of overlap)



## B. Modified retroviral components (overlap 1 reduced, overlap 2 and 3 eliminated)



## C. Modified retroviral components (overlap 1-3 eliminated)



## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 30763 A (VIAGENE INC) 16 November 1995 see page 28, line 33 - page 29, line 11 ---	1-38
X	WO 95 31566 A (VIAGENE INC) 23 November 1995 see page 29, line 31 - page 30, line 8 ---	1-38
X	WO 94 29438 A (CELL GENESYS INC) 22 December 1994	21
Y	see page 11, line 16 - line 26 --- -/--	1-20, 22-38

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

6 August 1997

Date of mailing of the international search report

14.08.97

Name and mailing address of the ISA

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Authorized officer

Hornig, H

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOTECHNIQUES, vol. 7, no. 9, October 1989, pages 980-990, XP000606889 MILLER A D ET AL: "IMPROVED RETROVIRAL VECTORS FOR GENE TRANSFER AND EXPRESSION" see page 982, right-hand column, line 2 - line 14 ---	1-20, 22-38
A	WO 96 07749 A (CHIRON VIAGENE INC) 14 March 1996 see the whole document ---	1-38
A	WO 92 05266 A (VIAGENE INC) 2 April 1992 cited in the application see the whole document ---	1-38
A	WO 89 07150 A (UNIV COLUMBIA) 10 August 1989 see the whole document ---	1-38
A	J. VIROLOGY, vol. 61, no. 5, 19 May 1987, AM.SOC.MICROBIOL.,WASHINGTON,US, pages 1639-1646, XP002036903 M.A. BENDER ET AL.: "Evidence that the packaging signal of Moloney Murine Leukemia Virus extends into the gag region" see the whole document ---	1-38
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 7, no. 5, 1 May 1987, pages 1797-1806, XP002036904 BOSSELMAN R A ET AL: "REPLICATION-DEFECTIVE CHIMERIC HELPER PROVIRUSES AND FACTORS AFFECTING GENERATION OF COMPETENT VIRUS: EXPRESSION OF MOLONEY MURINA LEUKEMIA VIRUS STRUCTURAL GENES VIA THE METALLOTHIONEIN PROMOTER" cited in the application see the whole document ---	1-38
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 85, no. 17, 1 September 1988, pages 6460-6464, XP002036905 DANOS O ET AL: "SAFE AND EFFICIENT GENERATION OF RECOMBINANT RETROVIRUSES WITH AMPHOTROPIC AND ECOTROPIC HOST RANGES" cited in the application see the whole document ---	1-38

-/--

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JOURNAL OF VIROLOGY, vol. 62, no. 4, 1 April 1988, pages 1120-1124, XP000562362 MARKOWITZ D ET AL: "A SAFE PACKAGING LINE FOR GENE TRANSFER: SEPARATING VIRAL GENES ON TWO DIFFERENT PLASMIDS" cited in the application see the whole document -----</p>	1-38

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/07697

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9530763 A	16-11-95	AU 2585395 A CA 2158977 A EP 0772689 A	29-11-95 10-11-95 14-05-97
WO 9531566 A	23-11-95	AU 2589695 A CA 2158926 A EP 0759087 A	05-12-95 14-11-95 26-02-97
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WO 9205266 A	02-04-92	AU 4798496 A AU 665176 B AU 8842491 A CA 2092195 A EP 0549723 A JP 6500923 T US 5591624 A	20-06-96 21-12-95 15-04-92 22-03-92 07-07-93 27-01-94 07-01-97
WO 8907150 A	10-08-89	AU 3069189 A CA 1315719 A US 5278056 A	25-08-89 06-04-93 11-01-94